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El Niño and Marine Iguana (*Amblyrhynchus cristatus*) Reproduction and Genetics

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Biology

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Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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DEDICATION

This work is dedicated to all action expanding upon unending truth and freedom for all.

CONTENTS

Approval Page iii

Acknowledgements iv

Dedication v

Table of Contents vi

List of Tables ix

List of Figures xi

List of Abbreviations xvi

Abstract xx

Chapters

1. Project Introduction 1
    - Introduction 1
    - Objectives Met 4
    - Significance 5
    - Relation to Current Knowledge and Long Term Goals 6

2. Methods and Procedures 8
    - Field Data Collection 8
        - Overview 8
        - Selection of Species 9
        - Choice of Study Population 10
        - General Methods 10
            - Physical Measurements 11
            - Permanent Marking of Animals 12
            - Blood Sampling 13
            - Systematic Behavioral Observations 13
    - Laboratory Work 18
        - Specific Molecular Marker Development Methods 19
            - Secondary Screening of Plasmids with DNA Inserts 21
            - Design and Testing of Primers 23
            - Characterization of Microsatellite Primers 25
                - PCR Conditions 25
                - High Resolution Spreadex® Gel Production 26
                - Gel Loading, Running, and Visualization 27
    - Statistical Analyses 28

3. Results and Interpretation 30
  Behavioral Observation Data 30
    Observer Uniformity 30
    Total Observed Population 36
    Density Patterns 45
    Movement Patterns 58
    Relative Intertidal Location 65
    Male Territoriality Observations 72
    Behavior Frequencies 74
    Grouping Tendency 82
    Behaviors Associated with Grouping 89
  Physical Measurement Data 106
    Population Means 106
    Paired Measurements 110
    Physical Traits and Later Observation / Survival 115
  Regression Analyses 121
    Behavior Ranking 121
    Predicting Behavior 124
    Predicting Number Nearby 126
    Predicting Survival 128
  Microsatellite Molecular Marker Data 130
    Primers Tested 130
    Allele Heterozygosity 133
    Potential Paternity Testing Value 135
    Allele Frequencies Associated With Physical Data 135
  A Program for Simulating Populations 139

4. Discussion 144

References 155

Appendices 160

Appendix A – Solutions and Reagents Used 160

Appendix B – Principle Behind Secondary Screening 165

Appendix C – Principles of PCR and Electrophoresis 172
    Principles of PCR 172
    Principles of Electrophoresis 178

Appendix D – Preparation and Use of Spreadex® Polymer NAB Gels 180

Appendix E – Microsatellite Primer Sets Tested 183

Appendix F – Permanent Identification Numbers Used 198

Appendix G – Basic Molecular Marker Development Methods ... 200
  - Extraction of DNA from Blood Sample ... 200
  - Preparation of Partial Genomic Library ... 202
    - DNA Fragments and Plasmid/Vector Ligation ... 203
    - Preparation of Cells for Insertion of DNA Fragments ... 206
  - Screening of Partial Genomic Library ... 210
    - Colony Growth and Non-Radioactive Detection ... 212

Appendix H – Instruction Manual for Diversion ... 216

TABLES

Table Page

Table 1. Behaviors observed and codes used during behavioral observations. 17

Table 2. Microsatellite primer set $MgCl_2$ and annealing temperature values used in PCR runs. 26

Table 3. Changes in measured means over one year for selected physical traits in three segments of the marine iguana population on Caamaño are shown. 109

Table 4. Changes in measured means over one year for selected physical traits in paired sets of male marine iguana data on Caamaño are shown...................112

Table 5. Changes in measured means over one year for selected physical traits in paired sets of female marine iguana data on Caamaño are shown. 113

Table 6. Changes in measured means over one year for selected physical traits in paired sets of hatchling marine iguana data on Caamaño are shown. 114

Table 7. Comparison of selected characteristics from initial measurements of male and female marine iguanas in 1997 on Caamaño, grouped by their last live observation date. 119

Table 8. Comparison of selected characteristics from initial measurements of hatchlings taken immediately after hatching in mid 1997 on Caamaño, grouped by their last live observation date. 120

Table 9. Microsatellite primer sets evaluated for usefulness in marine iguana paternity testing...131

Table 10. Comparison of observed and expected microsatellite allele heterozygosity between two studies of marine iguanas on Caamaño, Hardy-Weinberg equilibrium test, and linkage disequilibrium analysis within the population sample. 134

Table 11. Identification of statistically significant allele frequency differences between groups of territorial males, using individual microsatellite loci and then all loci combined, based upon physical characteristics and year of observation..137

Table 12. Approximate number of cycles (or generations) necessary in computer simulations before allelic frequencies between groups based on phenotype are observed to differ significantly..142

Table 13. Identification numbers used in permanently marking marine iguanas for observation. 199

FIGURES

Figure Page

Figure 1. Map of study islet with marked sections identified...15

Figure 2. Zones used for identification of intertidal location during behavioral observations. 16

Figure 3. Examples of identified resting positions. 18

Figure 4. Cumulative observation frequency of territorial male behaviors during the 1997 reproductive season, sorted by observer. 32

Figure 5. Cumulative observation frequency of female behaviors during the 1997 reproductive season, sorted by observer. 33

Figure 6. Cumulative observation frequency of hatchling behaviors immediately following hatching in 1997, sorted by observer..34

Figure 7. Cumulative observation frequency of all (male, female, and hatchling) marine iguana behaviors during the 1998 reproductive season, sorted by observer. 35

Figure 8. Total number of daily observations during the 1997 reproductive season........38

Figure 9. Daily total number of unique territorial males and percent of all territorial male observations that were of unique individuals during the 1997 reproductive season...39

Figure 10. Daily total number of unique females and percent of all female observations that were of unique individuals during the 1997 reproductive season...40

Figure 11. Total number of daily observations during May-June, 1997. 41

Figure 12. Daily total number of unique hatchlings and percent of all hatchling observations that were of unique individuals immediately following hatching in 1997...42

Figure 13. Total number of daily observations during the 1998 reproductive season......43

Figure 14. Daily total number of unique males, females, and hatchlings and percent of all male, female, and hatchling observations that were of unique individuals during the 1998 reproductive season...44

Figure 15. Territorial male density during the 1997 reproductive season. 47

Figure 16. Map of territorial male density within sections (A-O) during the 1997 reproductive season. 48

Figure 17. Female density during the 1997 reproductive season. 49

Figure 18. Hatchling density within sections immediately following hatching in 1997. 50

Figure 19. Territorial male density during the 1998 reproductive season. 51

Figure 20. Map of territorial male density within sections (A-O) during the 1998 reproductive season. 52

Figure 21. Female density during the 1998 reproductive season. 53

Figure 22. Map of female density within sections (A-O) during the 1998 reproductive season. 54

Figure 23. Ratio of total number of unique females to total number of unique territorial males observed within sections during the 1998 reproductive season. 55

Figure 24. Map of female to male density ratio within sections (A-O) during the 1998 reproductive season. 56

Figure 25. Hatchling density during the 1998 reproductive season. 57

Figure 26. Territorial male movement patterns during the 1997 reproductive season. 60

Figure 27. Female movement patterns during the 1997 reproductive season. 61

Figure 28. Hatchling movement patterns immediately following hatching in 1997. 62

Figure 29. Territorial male movement patterns during the 1998 reproductive season. 63

Figure 30. Female movement patterns during the 1998 reproductive season. 64

Figure 31. Relative intertidal location of territorial males from daily observations during the 1997 reproductive season. 66

Figure 32. Relative intertidal location of females from daily observations during the 1997 reproductive season. 67

Figure 33. Relative intertidal location of hatchlings from daily observations immediately following hatching in 1997. 68

Figure 34. Relative intertidal location of territorial males from daily observations during the 1998 reproductive season. 69

Figure 35. Relative intertidal location of females from daily observations during the 1998 reproductive season...70

Figure 36. Relative intertidal location of hatchlings from daily observations during the 1998 reproductive season...71

Figure 37. Daily percentage of marked (previously as territorial) males observers identified as displaying territorial behavior during the 1997 reproductive season...73

Figure 38. Territorial male behavior patterns as a proportion of all observed daily behaviors during the 1997 reproductive season...76

Figure 39. Female behavior patterns as a proportion of all observed daily behaviors during the 1997 reproductive season. 77

Figure 40. Hatchling behavior patterns as a proportion of all observed daily behaviors immediately following hatching in 1997.....................................78

Figure 41. Territorial male behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season...79

Figure 42. Female behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season. 80

Figure 43. Hatchling behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season...81

Figure 44. Time based pattern indicates percent of all territorial males observed during a single day that had a categorical number of females within 1 m during the 1997 reproductive season. 83

Figure 45. Time based pattern indicates percent of all females observed during a single day that had a categorical number of females within 1 m during the 1997 reproductive season...84

Figure 46. Time based pattern indicates percent of all hatchlings observed during a single day that had a categorical number of other iguanas within 1 m immediately following hatching in 1997. 85

Figure 47. Time based pattern indicates percent of all males observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. 86

Figure 48. Time based pattern indicates percent of all females observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. 87

Figure 49. Time based pattern indicates percent of all hatchlings observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. 88

Figure 50. Territorial male behavior patterns related to mean number of females within 1 m in daily observations during the 1997 reproductive season. 93

Figure 51. Territorial male behavior patterns related to proportion of all females seen within 1 m in daily observations during the 1997 reproductive season. 94

Figure 52. Female behavior patterns related to mean number of females within 1 m in daily observations during the 1997 reproductive season. 95

Figure 53. Female behavior patterns related to proportion of all females seen within 1 m in daily observations during the 1997 reproductive season. 96

Figure 54. Hatchling behavior patterns related to mean number of iguanas within 1 m in daily observations immediately following hatching in 1997. 97

Figure 55. Hatchling behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations immediately following hatching in 1997. 98

Figure 56. Territorial male behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. 99

Figure 57. Territorial male behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. 100

Figure 58. Female behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. 101

Figure 59. Female behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. 102

Figure 60. Hatchling behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. 103

Figure 61. Hatchling behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. 104

Figure 62. Mean ± SD of individuals within 1 m (female sized in 1997 and all in 1998) of territorial males or marked females in 1997 and 1998. 105

Figure 63. Mean number of individuals nearby associated with specific behaviors observed during the 1997 and 1998 reproductive seasons on Caamaño. ...123

Figure 64. Variables used in simulation for no periodic population declines. 140

Figure 65. Variables used in simulation for periodic population declines due to “sort events.” 141

Figure 66. Overview of partial genomic library secondary screening methods. 165

Figure 67. PCR product patterns obtained during secondary screening. 167

Figure 68. Potential primer binding sites during secondary screening PCR. 168

Figure 69. Examples of possible PCR products produced when “screening oligos” are used with “sequencing oligos” and a microsatellite repeat region is present within the inserted DNA. 169

Figure 70. Example of a gel documenting the various banding patterns identified in Figure 67. 170

Figure 71. Overview of polymerase chain reaction (PCR) cycles. 172

Figure 72. Example of a primer used in PCR. 173

Figure 73. Summary of polymerase chain reaction (PCR) for amplification of specific DNA fragments. 175

Figure 74. Overview of electrophoresis. 179

Figure 75. Overview of partial genomic library construction method used. 202

Figure 76. Overview of non-radioactive colony screening method used during microsatellite marker development. 211

ABBREVIATIONS

A	adenine
A_{xxx}	absorbance
Ag	agonistic, fighting
bp	base pair
C	Celsius, centigrade, cytosine
$CaCl_2$	calcium chloride
cm	centimeter
Cop	copulating
dH_2O	distilled water
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	eating
EDTA	ethylenediaminetetraacetate
ENSO	El Niño-Southern Oscillation
g	gram, acceleration of gravity
G	guanine
H_0	null hypothesis
H_E	expected heterozygosity
H_O	observed heterozygosity
Hb or HbC	headbobbing with mouth closed
HbO	headbobbing with mouth open

HCl	hydrochloric acid
HLL	hind leg length
hr.	hour
HW	head width
IPTG	isopropyl-[beta]-D-thiogalactopyranoside
kcal	kilocalorie
kg	kilograms
km	kilometer
l	liter
LBS	longest back spine
LiCl	lithium chloride
LSN	longest spine on neck
m	meter
M	moles per liter
mg	milligram
$MgCl_2$	magnesium chloride
min.	minute
ml	milliliter
mm	millimeter
mM	millimole
µg	microgram
µl	microliter
µJ	microjoule

n	sample size
NaCl	sodium chloride
Na_2EDTA	disodium ethylenediaminetetraacetate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
PCR	polymerase chain reaction
pH	potential of hydrogen
pmol	picomole
r or R1	resting prone, flat on ground
R or R2	resting with only head up
R or R3	resting propped up on front legs
RAPD	random amplified polymorphic DNA
rpm	revolutions per minute
SAP	shrimp alkaline phosphatase
SD	standard deviation
SDS	sodium dodecyl sulfate
sec.	second
Sm	swimming
SSC	standard saline citrate
SVL	snout-vent-length
SW	side-walking
T	thymine

TAE	tris acetate EDTA buffer
TE	tris-EDTA buffered solution
TEMED	N, N, N’, N’-tetramethylethylenediamine
TL	tail length
T_m	temperature at which 50% of a nucleic acid is in duplex with a complementary strand
UV	ultraviolet
V	volt
VNTR	variable number tandem repeat
Wa	walking
WT	weight
X-Gal	5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside
Y	C + T (C or T as oligonucleotide base in a sequence)

ABSTRACT

El Niño and Marine Iguana (*Amblyrhynchus cristatus*) Reproduction and Genetics

by

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Doctor of Philosophy, Graduate Program in Biology
Loma Linda University, December 2003
Ronald L. Carter, Chairman

Galápagos marine iguanas (*Amblyrhynchus cristatus*) have recently been described as the first "lek mating" reptiles. Female choice is often a dominant feature in explanations of lek development and/or maintenance. In order to examine survival and reproductive success, as well as search for clues to female choice, the marine iguana population on the small islet of Caamaño within the Galápagos of Ecuador was studied over two years (from the reproductive season of 1997 through the reproductive season of 1998). Physical measurement, behavior, and blood sample data were collected. Behavior of territorial males was correlated with number of other iguanas nearby. Due to an El Niño-Southern Oscillation (ENSO) that was in progress, large numbers died during the latter part of the observation period. Survival in females was linked to small size while survival in hatchlings was linked to large initial size. Microsatellite molecular markers were developed in order to allow paternity testing. Using strict parameters, projected accuracy of parental identification was 100% when one parent was known and 38% when neither parent was known. Examination of a sample of adult males (50) and females (10) indicated that many individual microsatellite loci had specific allele frequencies associated with phenotypic traits. In an effort to explain the results, a computer program was created in order to simulate a population with recurring population “booms and crashes” (similar to what marine iguanas experience during regular ENSO events). Tests indicate that when few microsatellite loci are evaluated, association of allele frequencies with phenotypic traits can occur within only a few generations, however, associations (length of longest spine on back, location, and year) found in the population sample when all 12 of the microsatellite loci were used are considered important, suggesting future interesting investigations.

PROJECT INTRODUCTION

Introduction

The Galápagos marine iguana (*Amblyrhynchus cristatus*) has recently been described as the first “lek mating” reptile. One typically invoked explanation of lek development and evolutionary stability is that of “female choice,” where females expend energy actively searching for a suitable mate. One means of determining the value of such models involves following the offspring of one breeding season through their growth and developmental stages. Analyses of offspring survival, physical characteristics, and reproductive success, if possible, are then used to determine the benefits each female receives through choice of a particular mate. Additional analyses of male characteristics (including activity, morphological features, and reproductive success) can be used to determine male quality and traits females might base selection on. This project included the collection of initial individual (male, female, and hatchling) characteristics (both measurements and behavior) for analyses as well as the production and characterization of the molecular markers necessary for parental assignment to offspring. This integrated use of behavior and molecular techniques has led to both a greater understanding of the dynamics of the mating system as well as assisted others interested in using molecular markers for paternity analysis in this species and others that are closely related.

Much has been written about the general life history and mating behavior of the marine iguana (*Amblyrhynchus cristatus*) of the Galápagos (Carpenter 1966, Trillmich 1983, Laurie 1990, Laurie & Brown 1990a, b). This primarily herbivorous lizard is only found living along rocky Galápagos island shores (Archipiélago de Colón, Ecuador).

During the greater part of the year, groups of male and female marine iguanas coexist peacefully. However, as the breeding season (December-February) approaches, adult males become increasingly territorial. There is often also a marked movement of adult males from their original locations (outside of the breeding season) to new sites (during the breeding season). Males on established territories battle to remove intruding males. Receptive females generally mate once each breeding season. The majority of copulations occur within established territories and insemination is most often linked to the territory's owner.

The mating system of the marine iguana has recently been categorized as “lek” (Wikelski 1996, Wikelski *et al.* 1996). This categorization follows many years of research and observation that produced conflicting opinions of the system's characteristics (Carpenter 1966, Trillmich 1983, Trillmich & Trillmich 1984, Wikelski *et al.* 1996). Multiple hypotheses have been proposed to explain original development and stability of lek mating systems (female preference - Bradbury 1981, hotspots - Bradbury & Gibson 1983, hotshots - Beehler & Foster 1988, black hole - Stillman *et al.* 1996). One consistent feature of all hypotheses is the need for “female choice.” Female choice is defined as any energetic activity knowingly or unknowingly engaged in by a female for the purposes of “selecting” which male or type of male to mate with. “Knowingly” and “unknowingly” can be alternatively expressed as “learned” vs. “genetically determined” behavior.

The “female preference” model identified by Bradbury (1981) uses the following simple rule to explain female choice of mate preference: Females prefer to mate with males that are territorial and clustered. This simple rule would drive males to group together and fight among themselves for territories. Males that could not compete with their peers would be excluded and not reproduce in high numbers.

Bradbury and Gibson's (1983) “hotspot” model is an extension of the female preference model and explains the location of male territories. In this model, clustering of male territories is determined by the sequential settling of males onto sites that are preferred by females. Active female choice is not as apparent and male-male interactions can influence reproductive success significantly.

“Hotshots,” in the model proposed by Beehler and Foster (1988), group together as in the female preference model, because females prefer to mate with males in groups. However, there is a further refinement of the rule that states females prefer to mate with dominant males in the groups. This places increased importance on the competition between males and requires a mechanism to determine dominance hierarchy by females.

Stillman *et al.* (1996) propose in their “black hole” model additional factors that may lead to females preferring grouped males. Mobile females are retained on (or decide to stay within) clustered male territories because there is some increased value or benefit over free-ranging movement. Some suggested benefits to remaining within a cluster of territories are: 1) easier sampling of mating partners, 2) avoidance of harassment, 3) decreased predation, or some other unidentified factor(s).

These descriptions of the principal components of the models point out the fact that each essentially differs from the others only in how or why females are making the choice. The work of this study is part of a larger project examining the reproductive advantage that might be obtained by females mating with specific males. Wagner (1998) points out the difference between female “choice” and female “preference” and suggests that multiple test methods are necessary in order to determine the extent and importance of the two for a population. However, his tests are based on experimental behavioral observations and manipulations. The work presented in this document expands knowledge of and adds tools enabling further examination of a population at both behavioral and genetic levels in order to infer the significance of female choice in the mating system.

Objectives Met

This study examined the physical and behavioral traits associated with territorial reproductive behavior in the marine iguana (*Amblyrhynchus cristatus*). Additionally, it produced molecular marker tools necessary for paternity analysis in the species. Fieldwork required three visits to the study site.

- Male Territoriality and Physical Traits: During the first visit to the study site, male territorial location and behavior were recorded. However, prior to behavioral observations, permanent marking and measurements of physical characteristics were completed, and a blood sample was taken from each territorial male. This information was helpful in determining territory structure and aided in identification of potential morphological characteristics of value to females in their assessment of male quality.
- Female Reproductive Activity: While mating was taking place during the first visit, females were identified, marked, measured, and a blood sample was taken following each observed copulation. Later, as nests were dug and eggs laid, unmarked females were captured, measured, and a blood sample was taken. This

provided maternal data necessary for later classification as well as a measure of egg-deposition timing and potential.

- Hatchling Identification: During the second visit, hatchlings of the previously monitored mating season were captured as they emerged from nests. Physical measurements and a blood sample were taken. This was followed by permanent marking for later identification. Data collected enabled paternity analysis testing and preliminary survival data have been calculated.
- Survival Rates: During the third visit (one year after the initial reproductive season), survival rate and growth of marked hatchlings were assessed. Additionally, during the second and third visits, marked male and female survivors were noted. Observation and measurement data have been used to analyze individual fitness as well as to help identify characteristics that lead to a higher probability of survival.
- Development of Markers for Paternity Analysis: In order to determine reproductive success and the interrelationship of male and female traits on offspring viability, accurate matching of parents with offspring is necessary. To accomplish this, microsatellite molecular markers were developed for DNA fingerprint analysis.

Significance

Many previous studies have examined the mating system of the marine iguana (*Amblyrhynchus cristatus*). However, these have dealt primarily with behavioral observations, morphological analyses, and ecological evaluations of reproductive success

(Carpenter 1966, Dellinger 1996, Laurie 1990, Laurie & Brown 1990a, b, Trillmich 1983, Wikelski & Baurle 1996, Wikelski *et al.* 1996, Wikelski & Trillmich 1997, Wikelski *et al.* 2001, Partecke *et al.* 2002, Hayes *et al.* 2004). This study initiated the integrated use of both behavioral observations and molecular markers in determining individual reproductive success in this species. Previous studies have used biochemical assays (Higgins & Rand 1974, Higgins *et al.* 1974, Higgins & Rand 1975, Higgins 1977, Higgins 1978, Higgins 1980) or molecular markers (Rassmann 1997, Rassmann *et al.* 1997a, b) in analysis of marine iguana populations, but none have developed and used techniques precise enough to determine paternity with certainty. A combined use of behavioral and molecular analyses provides a more accurate picture of the mating system (female selection criteria and reproductive success, territorial male physical and behavioral characteristics and their effects on reproductive success, offspring developmental characteristics and their dependence upon parentage, and breeding male and female genetic characteristics/relatedness) than either of the methods can provide separately.

Relation to Current Knowledge and Long-Term Goals

This work increases the current knowledge of marine iguana reproductive behavior and success in several ways. First, additional information concerning male territorial behavior (site fidelity, activity while on site, copulatory success rate) during the mating season was gathered. This information combined with that of previous studies enables comparison of behavior at different times (climatologically) and locations (geographically). Second, further information concerning female nesting activity (time, location, and guard behavior) as well as female physical condition at nesting was gathered and analyzed. Comparison of these observations with previous studies during multiple years and at different sites will be of value to park managers. Third, hatchling physical and behavioral characteristics following emergence from the nest has been compared to data from other studies. Fourth, permanent marking of males, females, and hatchlings facilitates later work monitoring survival rate. The recapturing conducted for this study is only the beginning of the valuable data that might be obtained by other researchers for long-term analysis of the population. Fifth, development of the molecular tools necessary for paternity analysis for this species is potentially of great value to those who plan to conduct further research with this or a closely related species (such as the Galápagos land iguana, *Conolophus* sp.) Additionally, combination of field observations and finer scale laboratory analyses provides a more valuable, persuasive, accurate, and complete picture of what is happening in the world and is a model worthy of incorporation into other work.

METHODS AND PROCEDURES

Field Data Collection

Marking, blood sampling, and measurement methods described here have been successfully used in the past and it has been determined that a minimal amount of pain and suffering is inflicted (Dellinger & von Hegel 1990, Laurie 1990, Laurie & Brown 1990a, b, Wikelski 1994). Following these same methods ensured both humane treatment and provided comparable results.

Overview

This project comprised two distinct phases. First completed was original marking, sampling, and observation of all individuals studied in the population. The sampled population comprised the entire breeding assemblage on a small islet during one year. A one-year follow up re-sampling of all survivors was conducted. Following this collection of field data, lab work that had been initiated during time away from the field was continued in the form of a search for appropriate molecular markers necessary for paternity testing. Markers of choice for paternity testing are microsatellite loci (Queller *et al.* 1993, Pena & Chakraborty 1994, Ellegren et al 1995, O'Reilly & Wright 1995, Cooper *et al.* 1997, Double *et al.* 1997, Gullberg *et al.* 1997, Hughes & Deloach 1997). Use of microsatellite loci allows “fingerprint” profiles to be established for individuals followed by statistical comparison of variability and similarity between profiles or groups of profiles. In the selection of appropriate loci with characteristics necessary for paternity testing, additional loci were inevitably discovered that may provide useful information at a population or higher level, but are not appropriate for paternity analysis. These loci are reported for use in additional work.

Selection of Species

The marine iguana is the first described reptilian lek mating species and its study has several advantages over other lek species (in addition to simple interest in study of their unique way of life) as a drawing factor toward further study. In an analysis of a species' reproductive behavior (and for research in general), it is useful to have as large and complete a data set as possible. First, the animals are easily located and limited in distribution. They are only found along the shorelines of the Galápagos (Archipiélago de Colón) of Ecuador. Secondly, they are easy to capture and work with. Darwin's (1845) oft quoted assessment is quite accurate: “The rocks on the coast are abounded with great black lizards between three and four feet long; . . . It is a hideous-looking creature, of dirty black colour, stupid and sluggish in its movements. . . . They do not seem to have any notion of biting; . . .” While the specific descriptors used may not be agreed to by all, their implications provide an accurate portrait of their behavioral characteristics and general appearance within the habitat. Third, according to current knowledge, territorial males provide no resources (other than sperm) to attract females. This provided a good study subject when examining the potential value and implications of female reproductive choice. Number of variables that have to be worked with is reduced since males provide only genetic material and female energetic input following selection of nest site and egg-laying is limited to a short period of nest guarding. Fourth, throughout the year, including the breeding season, the majority of the iguanas' time is spent on the rocky beaches either sunning themselves or foraging within the intertidal zone, or in the case of territorial males during the breeding season, maintaining a territory principally above the area of highest tide and attempting to copulate. Male territories within this open and easily viewed region ensure behavioral observations are easily made. These characteristics make the marine iguana a species easily captured, measured, sampled, and observed. Because of the ease of data collection, a large sample size was used, potentially providing more accurate results and dependable conclusions.

Choice of Study Population

Site selection for this work was based upon examination of previously completed work on the small islet Caamaño (Carpenter 1966, Trillmich 1983) and discussion with Martin Wikelski (pers. comm.) The site has the advantages of being small and separated (app. 1 km) from a larger island, Isla Santa Cruz. The small study area ensured that a complete breeding assemblage could be sampled and observed systematically. Additionally, the islet is near the Charles Darwin Research Station (Estación Científica Charles Darwin) within Bahía Academy on Isla Santa Cruz. Such close proximity to the research station allowed ample logistic support.

General Methods

Data were gathered on the small islet of Caamaño located approximately 1 km south of Isla Santa Cruz within the Galápagos, bordering Bahía Academy. The islet's small size enabled collection of data from nearly all territorial males, egg-laying females, and offspring during one reproductive season. The following physical measurements and behavioral observations were collected.

Physical Measurements

- Gender - Gender was determined by visual observation or by cloacal probing (Dellinger & von Hegel 1990).
- Snout-vent-length - Measured along the belly from the tip of the snout to the opening of the cloaca (to the nearest mm).
- Tail length - Measured along the underside of the tail from the opening of the cloaca to the tip (to the nearest mm).
- Hind leg length - Measured from the center of the body axis between the legs to the tissue between the first and second toes while the leg was held at 90° to body axis (to the nearest mm).
- Head width - Measured at widest point of the head behind the eyes (to the nearest 0.1 mm).
- Weight - Weight to nearest 50 g for males and females and nearest 1 g for hatchlings.
- Longest neck spine - Measured length of the longest spine along the neck (to the nearest 0.1 mm).
- Longest back spine - Measured length of the longest spine along the back, for adult males only (to the nearest 0.1 mm).
- Number of neck spines - Counted number of neck spines/nubs along the neck, for hatchlings only.

- Cloaca depth - Measured depth of sex probe penetration, for hatchlings only (to the nearest 0.1 mm).
- Ticks - Number of ticks found over the entire body.
- Mites - Relative number of mites found around the neck of the animal. The scale used ranged from 0-3; with 0 representing very few or none and 3 indicating highest level with large patches of skin covered completely by the reddish parasite.
- Temperature - Hatchling temperature to the nearest 0.1°C taken just prior to running test. Temperature taken by holding electronic probe tip between hind leg and body.
- Speed/Time - Total time hatchling ran before stopping following experimental stimulation. Experimental stimulation involved holding the hatchling at a starting point, hitting a hammer to a metal plate two times, and then releasing the hatchling to run down a fenced runway on the third strike. If hatchling ran beyond runway, time stopped at runway exit.
- Distance - Distance hatchling ran before stopping (to the nearest 0.1 m). If hatchling ran beyond runway exit, length of runway (5.9 m) was recorded as distance.

Permanent Marking of Animals

Marking of individuals for observation and survival analysis entailed two procedures. First, each animal was permanently marked by branding with a hot wire. This practice has been used successfully in the past following determination of its safety and humanity (Laurie 1990, Laurie & Brown 1990a, b, Snell pers. comm., Wikelski pers. comm.) and provided permanent means of identification. Individuals were marked in three places (on both sides and the belly) with a given number. Hatchlings received an additional mark (a line lengthwise) on the underside of the tail as an additional means of identifying them as cohorts for long-term observation. Secondly, each animal had an identification number painted on its sides and a bit of paint was placed along the neck spines and the outer edges of the crown of the head. Painting individual iguanas to facilitate re-identification and observation has been determined to be safe and humane (Trillmich 1983, Wikelski 1994). Painted numbers remained legible for approximately 2-3 months, before flaking off or being shed (along with old layers of skin). All numbers used for permanent marking and identification are listed in Table 13, located in Appendix F (page 198).

Blood Sampling

A blood sample was collected from each individual upon initial capture. Blood was collected from a caudal vessel at the time of measurement and permanent marking. A 1 ml sample was taken from adult males and females while a 0.25 ml sample was taken from hatchlings. Blood was preserved in Queen’s Lysis Buffer (see Appendix A, beginning on page 160).

Systematic Behavioral Observations

Behavioral observations were made while moving around the islet in either a clockwise or counterclockwise direction beginning within a randomly selected “section” (see Figure 1). An ordered route ensured that all locations were observed with equal frequency. Reduced disturbance of animal activity and increased efficiency was obtained by following a sequential route rather than choosing random sections to observe and having to “jump” from one randomly selected section to the next. (The extra observer motion necessary for absolutely random observations would have disturbed many more animals.) A sequential route ensured that as much information was collected as possible in as timely and efficient a manner as possible.

During observations, every effort was made to ensure as little disturbance as possible was introduced by the observer. When necessary, binoculars were used for identification of individual iguanas. Observations took place at all times during daylight hours (when iguanas are active) in an effort to obtain representative data. The following data concerning location, behavior, time and date of observation, observer, iguana identification number, and gender were collected:

- Section - Division of islet shoreline or land region where the individual and behavior was observed. Sections were arranged in approximately 30 m lengths. Figure 1 shows a drawing of the islet with sections marked.

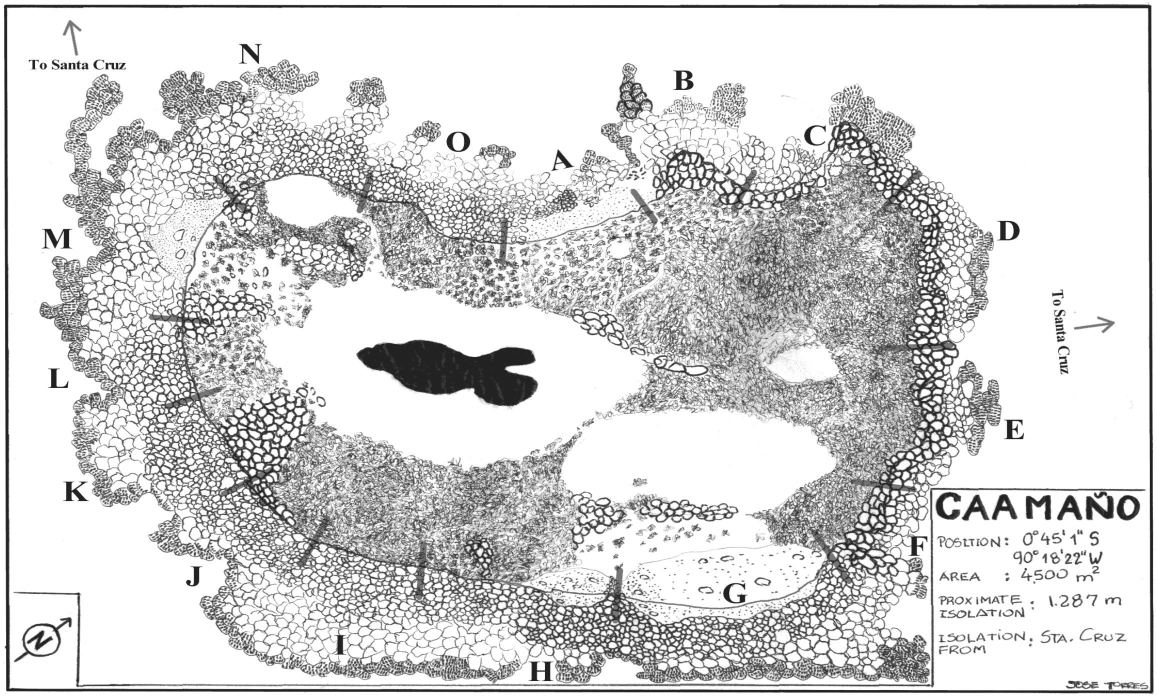


Figure 1. Map of study islet with marked sections identified.

- Zone - Location based on intertidal proximity. Distinctions ranged in four increments from low intertidal to high within the bushes above the reaches of high tide. Arrangement of zones is illustrated in Figure 2.

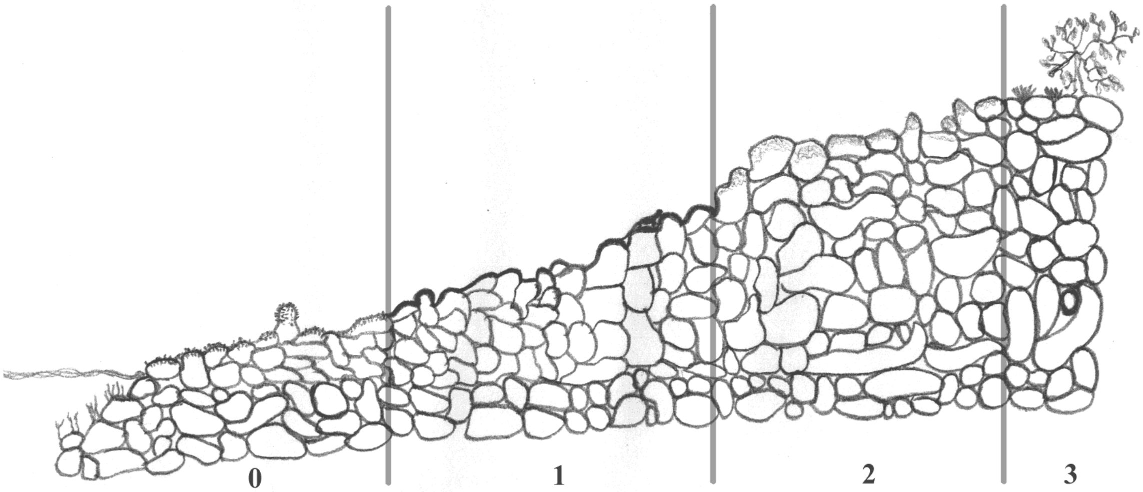


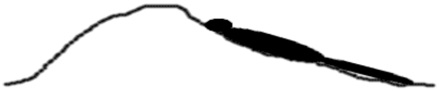
Figure 2. Zones used for identification of intertidal location during behavioral observations. Zone 0 begins where barnacles are found on the rocks. Zone 1 encompasses the darker rocks always washed over and cleaned by water but exposed during most low tides. Zone 2 is above most high tide ranges where rocks are more gray and white, being covered with feces. Zone 3 is high up where terrestrial plants grow.

- Females in 1 m - Number of female sized iguanas located within 1 m radius of an adult male (recorded for January-March, 1997).
- Number in 1 m - Total number of iguanas located within 1 m radius of the observed iguana (recorded in May-June, 1997 and January-February, 1998 instead of “Females in 1 m”).
- Number marked - Total number of iguanas identified as marked within a 1 m radius of the observed iguana.
- Territorial/Not Territorial - Evaluation of whether a male appeared to be territorial or not territorial during observation (recorded only for males in January-March, 1997).

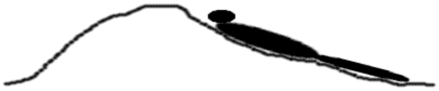
- ID of nearest male - Identification number of the nearest marked male, within 10 m (recorded in January-February, 1998).
- Behavior - Observed behavior of individual. The most commonly observed behaviors were identified and given short codes. Additional comments on location or behavior were recorded as necessary (such as "headbob" counts for territorial males). Table 1 lists abbreviation and description for each behavior identified during observations. Figure 3 provides examples of the three types of resting positions identified.

Table 1. Behaviors observed and codes used during behavioral observations.

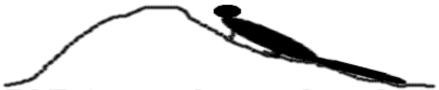
Code	Description
Hb	headbob
HbO	headbob with open mouth
Wa	walking
R1 = r	resting (prone/flat on the ground/rocks)
R2 = R	resting (just head up)
R3 = R	resting (propped up on front legs)
Ag	agonistic/fighting
Cop	copulating
SW	sidewalking
E	eating
Sm	swimming
D	digging/in nest
C	covering nest



R1/r (prone)



R2/R (just head up)



R3/R (propped up on front feet)

Figure 3. Examples of identified resting positions.

Laboratory Work

Paternity analysis has been performed using RAPD (random amplified polymorphic DNA, Tegelström and Höggren 1994) and VNTR (variable number tandem repeat, O'Reilly and Wright 1995) methods; however, such analyses are often performed on populations where one parent is known and/or sample size is small. The multiple sites amplified and visualized by these methods make allele frequency hard to determine. Additionally, due to the large number of bands involved, accurate scoring of individual fingerprints is difficult. Because of their great specificity (single location within the genome), microsatellite markers are relatively easy to work with once developed. Microsatellite molecular markers were discovered following the basic guidelines outlined by Glenn (1998) and Estoup and Turgeon (1996). These protocols were selected because of their wide use and common application toward microsatellite discovery in many eukaryotic organisms. Once identified, markers were tested on a sample of the territorial males (*n*=50) and breeding females (*n*=10) in order to determine marker variability and informative qualities. Markers with highest variability (largest number of alleles) within the population have been statistically analyzed and reported as useful in paternity analysis. Other markers with sub-optimal variability are reported as potentially useful in broad comparisons between separate populations or within related species (Rassmann 1997, Rassmann *et al.* 1997a).

Specific Molecular Marker Development Methods

The following is a description of the steps followed in identifying and screening microsatellite molecular markers for the Galápagos marine iguana (*Amblyrhynchus cristatus*). Briefly, the major steps in the microsatellite molecular marker development process were:

- Extract DNA from blood sample.
- Prepare or obtain competent cells (bacterial cells that DNA may easily be inserted into).
- Prepare vector for DNA insertion - cut and dephosphorylate.
- Prepare insert DNA for attachment to vector - cut and size-select.
- Ligate size-selected insert DNA into vector.
- Transform competent cells with ligation products (vector with selected DNA inserted).
- Check insert size and transformation efficiency, re-transform and plate bacterial colonies if necessary.
- Lift/transfer colonies onto filters.
- Prepare probe (that will bind to specific sites within the inserted DNA).

- Hybridize probe (radioactive or colorimetric) to colonies on filters.
- Expose filters to X-ray film (radioactive labels) or chromogenic antigen.
- Select positive colonies and check for inserts.
- Isolate plasmid DNA for sequencing.
- Perform secondary screening to further determine DNA insert properties.
- Sequence selected inserts.
- Design primers for repeat region of DNA insert.
- Optimize PCR conditions for primer use.
- Screen for allelic variation (60 individuals).

Standard methods were used during initial primer development steps. Detailed methods are provided in Appendix G (beginning on page 200). Prior to sequencing, an additional “secondary screening” procedure was performed.

Secondary Screening of Plasmids with DNA Inserts

In order to select plasmids with inserted DNA to sequence that would provide the most valuable information, a "secondary screening" procedure was developed. The principles behind this procedure are outlined in Appendix B (page 165), where a graphical overview is also available.

Two separate PCR reactions were run for each extracted plasmid DNA sample.

1. PCR using the same primers used when forward and reverse sequencing reactions are performed – "sequencing primers" called 21M13 and Reverse 2 for pUC19.
2. Another PCR using the "sequencing primers" along with all six of the synthesized (unlabeled) oligos previously used for hybridization and detection of microsatellite repeat sequences.

PCR was performed using the following recipe prepared on ice (for nine 10 µl reactions):

54 µl [46 µl - if additional oligos used] PCR grade water.

7.5 µl $MgCl_2$ (25 mM stock) - 2 mM final concentration[1].

9 µl 10x Buffer[1].

9 µl dNTP's[2].

0.5 µl upper "sequencing primer" (21M13)[3,5].

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[1] Provided with Promega *Taq* DNA Polymerase

[2] Purchased from Epicentre as a premixed stock solution containing 2.5 mM of each dNTP

0.5 µl lower “sequencing primer” (Reverse 2)[3,5].

[If used, 1.3 µl of each of the 6 synthesized oligos[4,5] (see page 212) containing microsatellite repeats - 7.8 µl total.]

1 µl *Taq*[1].

9 µl of the above “master mix” and 1 µl plasmid DNA were put into each sample tube.

Each sample was then covered with 20 µl mineral oil (to prevent evaporation during PCR).

PCR was run using the following program:

45 cycles of . . .

94°C for 45 sec.

60°C for 1 min.

72°C for 1 min.

72°C for 9 more min.

Cooled down and held at 4°C.

PCR products (see Figure 70, page 170 in Appendix B, for an example) were run as pairs on an 8% polyacrylamide (16 cm long) gel for 1.5 hr. at 300 V. Pairs in which the PCR samples containing the oligonucleotide repeat sequences as primers had shorter products clearly visible were considered positive, indicating that a repeat sequence was located within the inserted DNA of the

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[3] Diluted in TE to a concentration of 250 pmol/µl

[4] Diluted in TE to a concentration of 100 pmol/µl

[5] All oligonucleotide primers synthesized by Genosys

plasmid. (See Appendix B, page 165, for an explanation of the reasoning behind the procedure.)

Selection of Plasmids to Send Out for Sequencing:

PCR data for each extracted plasmid were entered into a spreadsheet and sorted by total length of DNA insert (the size of the product produced when only "sequencing primers" were used). Plasmids that had products between 300-1000 were considered preferable (since it is fairly certain that sequencing in both directions will cover the entire inserted DNA sequence); however, up to 1500 bp was considered acceptable. Those DNA inserts that looked like the repeat sequence was in the middle (i.e. they had a shorter PCR product that was about half the size of the entire insert) or at least 100-200 bp from either end of the insert were believed to have the highest probability of yielding good sequence data that would allow ample room on either side of the microsatellite repeat to design functional primers.

Plasmids chosen for sequencing were dried in a facility-preferred quantity using a SpeedVac (centrifuge with a vacuum applied) and sent to California State University – Northridge to be sequenced.

Design and Testing of Primers

PCR primers were designed from the sequences received using the Macintosh version of Oligo 4.0 from Molecular Biology Insights. Characteristics selected for in primers included:

- Small difference in template-primer T_m, less that 35°C. (T_m is the temperature at which 50% of a nucleic acid is in duplex with a complementary strand.)
- Relatively low 3'-terminal stability, so that the 3'-pentamer delta-G is more positive than -9 kcal/mol. (delta-G is a measurement of free energy. DNA is more stable [held together more tightly with more stable bonds] when delta-G is more negative.)
- A higher proportion of GC-content on the 5'-end of the primer is desirable.
- Small difference between the T_m of the primers. (A large difference in T_m between primers can lead to the primer with the higher T_m binding nonspecifically at the temperature necessary for the other primer to bind.)
- Dimer formation (binding) between primers and themselves or to each other is to be avoided, especially when the delta-G value is quite negative.
- Hairpin formation (a primer folding around and binding to itself) inhibits activity and is to be avoided, especially when the delta-G value is quite negative.
- Primers with approximately 50% GC-content are preferred.
- T at the 3'-end may lead to more frequent mis-priming than A, G, or C.
- Since low annealing temperatures are more likely to produce non-specific products, primers with higher melting temperatures allowing high annealing temperatures are beneficial.

- It is best to design primers as close to the microsatellite repeat as possible. This reduces the size of the PCR product produced and makes separation and analysis of the product on gels faster and more accurate.

Each set of developed primers was optimized for PCR product specificity (working to reduce presence of additional bands outside the sequenced product size range) by altering PCR annealing temperature and $MgCl_2$ concentration. The following section deals more specifically with recipes used and conditions of PCR.

Characterization of Microsatellite Primers

PCR conditions

PCR conditions while running multiple samples were generally the same except for changes in $MgCl_2$ concentration and annealing temperature. Below is the standard recipe utilized. Table 2 contains specific information concerning $MgCl_2$ concentration and annealing temperature used for each microsatellite primer set.

Standard PCR protocol (for 60 samples) – mixed on ice:

- Put 9 µl of PCR “master mix” (see Appendix A, page 162) in each of 60 1.5 ml microfuge tubes.
- Added 1 µl sample DNA to each microfuge tube with “master mix”.
- Layered 10 µl mineral oil (filtered and autoclaved) over solution in the microfuge tube.
- PCR cycles set to 35 cycles of . . .
    - 94°C 45 sec.
    - 54°C 1 min.

    - 72°C 1 min.

- Followed by . . .
    - 72°C 9 min.
    - Hold (store) at 4°C.

Table 2. Microsatellite primer set $MgCl_2$ and annealing temperature values used in PCR runs. See Appendix E (page 183) for additional sequence and primer information.

Microsatellite Primer ID / Repeat Type(s)		$MgCl_2$	Annealing Temperature
14(2)	$(TG)_{11}$	2.07 mM	56ºC
22	$(AC)_{11}$	2.76 mM	50ºC
30	$(TG)_{12}$	2.76 mM	50ºC
42	$(AC)_8$,$(AC)_4$	2.76 mM	58ºC
50	$(CT)_4(GT)_{15}$	2.76 mM	54ºC
119/147	$(TG)_{11}$	2.09 mM	45ºC
196	$(AT)_7(GT)_{13}$	2.09 mM	50ºC
2nd Am(GT)-2	$(TG)_{15}$	2.07 mM	48ºC
Am(GT)-4	$(TG)_9$, $(CA)_4$, $(GA)_3$, $(CTTCT)_3$	2.07 mM	46ºC
CCF-4/70		2.76 mM	56ºC
Co-18A	$(CTAT)_n$, $(CCAT)_n$	2.07 mM	53ºC
Co-18B	$(CTT)_n$, $(CCA)_2$	2.07 mM	53ºC

High resolution Spreadex® gel production

Below is the standard Spreadex® gel recipe for four 5% 0.5 mm thick polyacrylamide gels (using plates for the Hoefer Scientific Instruments SE 600 Series vertical slab gel unit and 0.5 mm thick spacers and combs cut from plastic craft sheeting purchased from Walmart). See Appendix D (page 180) for additional background information on Spreadex® gels.

- 8 ml 30% acrylamide.
- 240 µl 10% ammonium persulfate.
- 4.8 ml 10x Spreadex® Polymer NAB (from Elchrom Scientific).

- 960 µl 50x TAE.
- Up to 48 ml total volume with dH_2O and mixed well.
- 24 µl TEMED, mixed by swirling.
- Poured into plates up to just below (approximately 0.5 cm below) where the bottoms of the teeth for the comb forming the wells will go with a syringe.
- Layered 50% methanol carefully (in order to prevent mixing of methanol with gel) over the top of the 5% Spreadex® gel solution.
- Allowed to solidify for 15-20 minutes and mixed up 4% solution for top of gel.
- 2680 µl 30% acrylamide.
- 100 µl 10% ammonium persulfate.
- 400 µl 50x TAE.
- Up to 20 ml total volume with dH_2O and mixed well.
- Removed methanol from top of gel.
- Rinsed top of gel with 4% acrylamide solution (that had no TEMED added yet).
- Added 10 µl TEMED to remaining 4% acrylamide solution and mixed by swirling.
- Poured 4% acrylamide solution using a syringe.
- Placed comb to form wells in top 4% gel solution.
- Allowed to solidify for at least 1 hr.

Gel loading, running, and visualization

After they had solidified, gels were “pre-run” for 1 hr. at 300 V in 1x TAE running buffer. This was done in order to help to equalize any possible buffer imbalance between the gel and running buffer so that separation would be as uniform as possible. Each gel rig allowed up to two gels to be run.

Each 10 µl PCR product was mixed with 3 µl type III gel-loading buffer in the same tube PCR had been performed in. Molecular weight ladder (25 bp DNA Step Ladder from Promega) was mixed for use in 3 lanes per gel by combining 4.5 µl gel-loading buffer and 4.5 µl ladder. Each well/lane was loaded with 3-5 µl of the PCR product and dye mix (except for the outside two lanes that were generally left empty, since products close to the edge do not run as evenly) or with the molecular weight marker (generally at each end and in the middle), being sure to alter marker locations so that individual gels could be identified by ladder location.

Gels were run at 300 V for a period that had been experimentally determined to get the fragment size of interest to within 2-4 cm of the bottom of the gel. After electrophoresis had finished, gels were removed and stained 20-25 min. with ethidium bromide solution (in dH_2O) and de-stained in dH_2O for 10-15 min.

Visualization of stained gels was done using an Alpha Innotech IS-1000 Digital Imaging System. The system allowed photos as well as calculations of molecular weight. Linear best fit calculation settings were used when determining fragment length based upon molecular weight markers run in each gel.

Statistical Analyses

Data were stored, organized, and sorted using Microsoft® Excel 2000 (9.0.4402 SR-1). Behavioral analyses were done using SPSS for Windows 11.0.0 (19 Sep 2001).

Analyses of molecular data were completed using Arlequin 2.001 (Schneider *et al.* 2000) and CERVUS 2.0 (Marshall *et al.* 1998).

RESULTS AND INTERPRETATION

Behavioral Observation Data

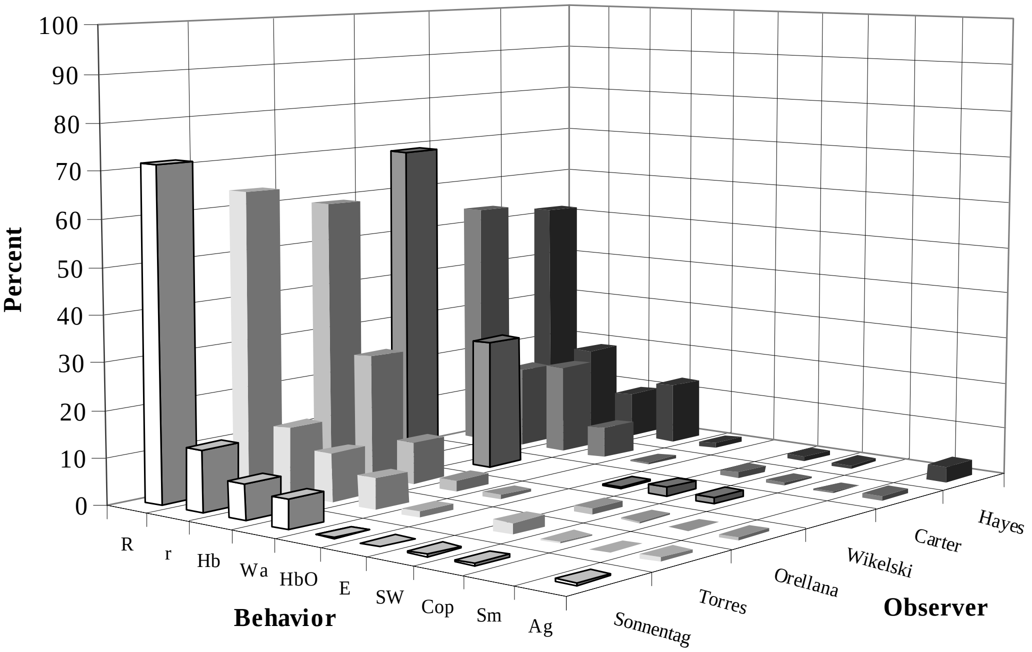
Behavioral observation data were entered into a spreadsheet (Microsoft® Excel 2000) and then sorted, tabulated, and plotted to provide descriptive statistics and to detect possible trends. Empty cells within tables and charts may be assumed to contain zeros. Zeros were removed to make 3-D charts easier to interpret. Tables were included below most charts in order for values hidden to be included in visual interpretation. All original data are included with this document on a CD/DVD for restructuring and further analysis at the reader's discretion.

Observer Uniformity

High observer correlation in behavioral data collection is displayed in the following four figures showing relative frequency of behavioral observations recorded by different individuals during the three observation periods. Behaviors identified are listed in Table 1 and Figure 3, beginning on page 17. Due to similarity of recorded behavior frequencies (Figure 4), all data were included in plotted graphs. Exceptions to overall similarity, such as for female behaviors observed during the reproductive season of 1997, when only three out of six observers saw marked females (Figure 5), and Daniel's observations of hatchling behavior later in 1997 (Figure 6), are considered to be due to extremely few observations and thus not likely to impact overall plots. However, the reader is advised to be mindful of this information during interpretation of later charts.

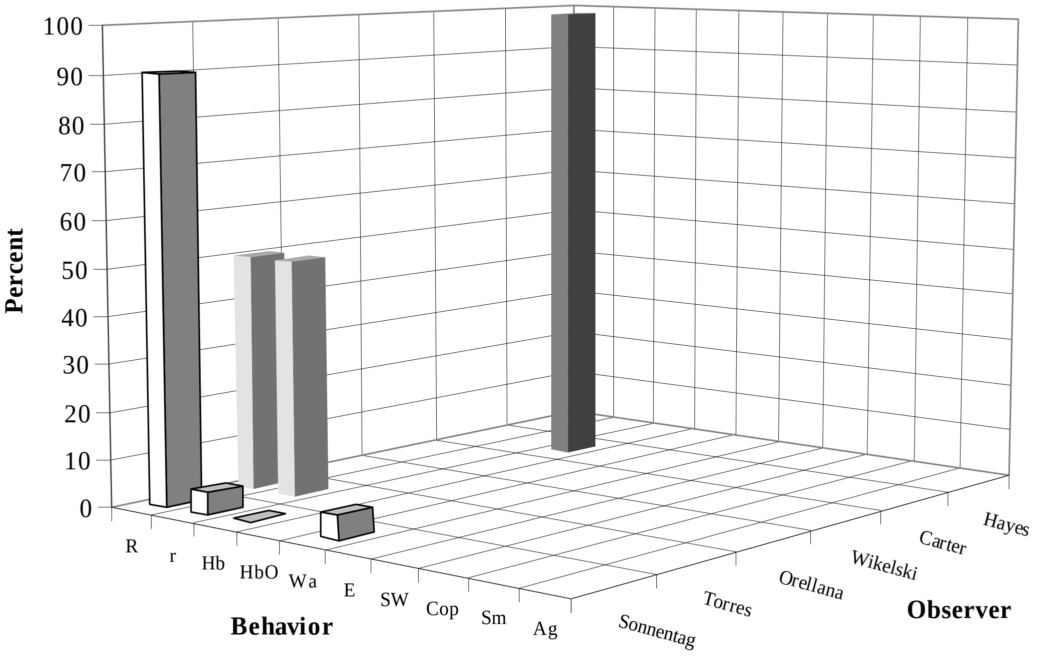
Percentage of time spent in various forms of "rest" was greater than that observed for any other activity. Rest in what is interpreted as the most "alert" or "attentive" position, R3 or R (see behavioral definitions, in Figure 3 on page 18, for graphical examples), was most commonly observed. This most common position may also serve as one of the more effective means of thermoregulation as well. During daylight hours, heating and cooling might be effected as position, solar angle, and cooling winds are taken into account. Specific observations necessary in order to answer this question with more certainty were not made.

Apparent change in frequency of observed rest positions between 1997 and 1998 may be partially due to change in health or, alternatively, appear as a result of a change in behavioral recording methods. Prior to observation of hatchlings in 1997, only two rest positions were noted (R and r, see Figure 3 on page 18), while later three positions were recorded. For this reason, no conclusion can be drawn concerning resting position and physical condition. However, when values for R in 1997 are compared with values for R3+R2 in 1998, they appear to be roughly equal. The most striking difference in overall behavior observed between the two reproductive years was lack of most observed agonistic and pre-copulatory behavior in 1998 (Figure 7). Some headbobbing and headbobbing with open mouth were observed, but such observations were comparatively rare in 1998.



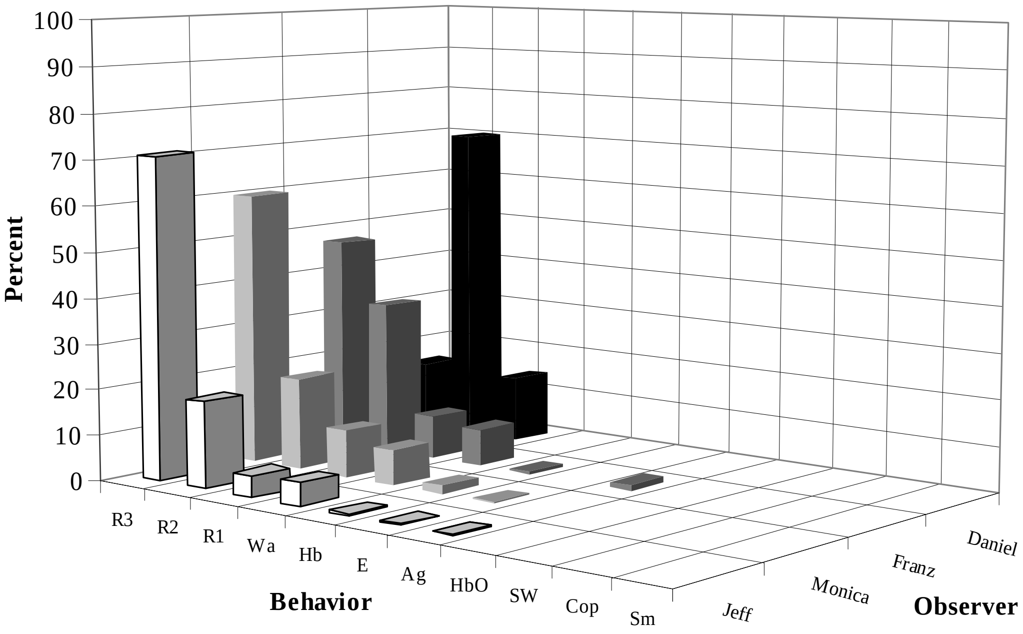
	R	r	Hb	Wa	HbO	E	SW	Cop	Sm	Ag
☐ Sonnentag	71.4	13.2	7.8	6.2	0.1	0.1	0.4	0.5		0.5
░ Torres	63.8	14.2	10.3	6.6	1.4		2.4	0.3	0.1	0.8
▒ Orellana	59.1	26.6	9.0	2.3	0.8		1.2	0.3	0.2	0.6
▣ Wikelski	68.9		27.9			0.3	1.7	1.2		
▓ Carter	53.6	17.4	19.1	6.6	0.4		1.2	0.5	0.2	0.9
■ Hayes	51.7	18.9	9.8	13.3	1.0		1.0	0.7		3.5

Figure 4. Cumulative observation frequency of territorial male behaviors during the 1997 reproductive season, sorted by observer. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, r-resting, R-resting, Wa-walking. Numbers in table are rounded off.



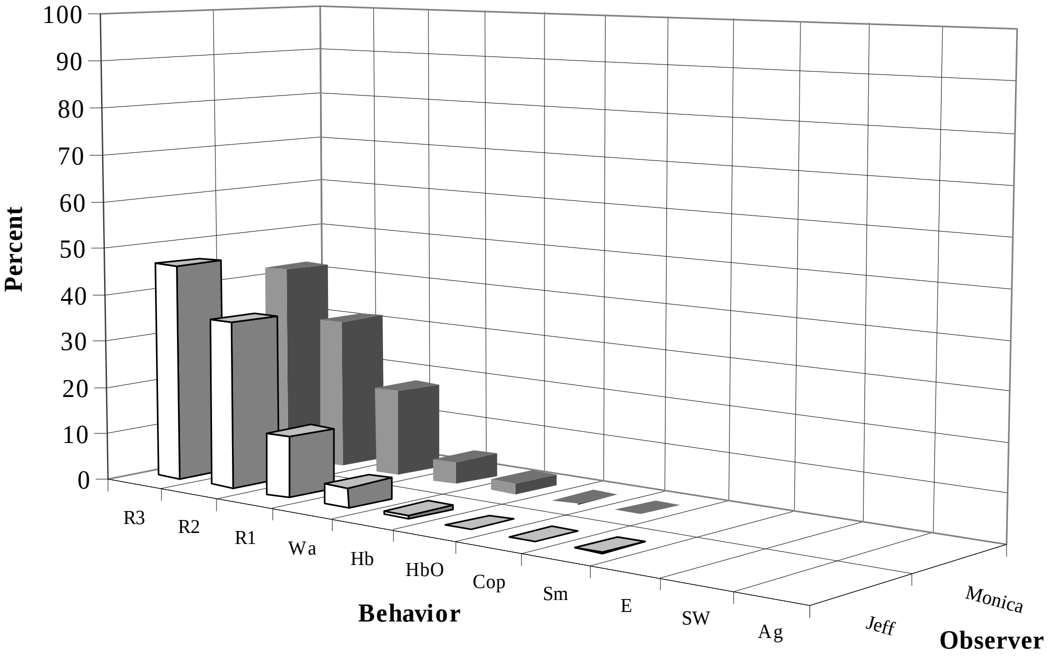
	R	r	Hb	HbO	Wa	E	SW	Cop	Sm	Ag
□ Sonnentag	90.2	4.6	0.2		5.0					
■ Torres	50.0	50.0								
■ Orellana										
■ Wikelski										
■ Carter			100.0							
■ Hayes										

Figure 5. Cumulative observation frequency of female behaviors during the 1997 reproductive season, sorted by observer. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, r-resting, R-resting, Wa-walking. Numbers in table are rounded off.



	R3	R2	R1	Wa	Hb	E	Ag	HbO	SW	Cop	Sm
□ Jeff	70.8	18.9	4.6	5.0	0.4	0.2	0.1				
▨ Monica	59.9	20.2	10.2	7.6	1.8	0.3					
▩ Franz	46.8	33.5	9.5	8.2	0.6		1.3				
■ Daniel	15.1	70.4	14.5								

Figure 6. Cumulative observation frequency of hatchling behaviors immediately following hatching in 1997, sorted by observer. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



	R3	R2	R1	Wa	Hb	HbO	Cop	Sm	E	SW	Ag
☐ Jeff	46.2	35.5	13.0	4.3	0.8	0.1	0.1	0.1			
■ Monica	42.3	32.1	18.3	4.7	2.4	0.1	0.1				

Figure 7. Cumulative observation frequency of all (male, female, and hatchling) marine iguana behaviors during the 1998 reproductive season, sorted by observer. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.

Total Observed Population

The following seven figures (Figure 8 - Figure 14) show total number of daily observations followed by total number of unique individuals observed each day, with percentage of all pooled daily observations that were of unique individuals. Both values are shown so that days having fewer observations may be identified and kept in mind while evaluating the contents of later figures.

Figure 9 shows one of the clearest trends detected throughout all observational periods. Nearly all marked territorial males were observed up through the 15th day of observation. However, from that point on there was a steady decline in the number of territorial males observed. During this later period of declining numbers, marked males were seen on Caamaño in areas far from their traditional territory. Later personal observations and reports of others, found marked territorial males on the shores of Isla Santa Cruz, approximately one to two kilometers away. Low total unique territorial male observations (and corresponding high percentage of observations of unique individuals) during the first two days and later on days 11 and 18 are attributed to reduced time spent making observations on those days.

Total daily observations of females plotted in Figure 10 does not identify any trend. Few females were marked when observations during the 1997 reproductive season started. Females were only marked when they were observed copulating. Between days 30 and 56, nest digging and egg laying began and many more females were marked. However, few observations were made after the large amount of marking took place.

During the second visit to Caamaño, hatchlings resulting from the previous few months' reproductive season were captured and marked as soon as possible, as shown in

Figure 12. Because of more hatchlings being marked, the general trend is upward each day with respect to the total number of unique individuals observed. Some low numbers are attributable to relatively few observations being made (see days 17-21 of Figure 12).

Information for the 1998 visit during the reproductive period is shown in Figure 14. Number of territorial males observed during the period was much less than the corresponding period in 1997. Between the first visit in 1997 and the third visit in 1998, an ongoing El Niño-Southern Oscillation (ENSO) event had been taking place. However, during the observation period in 1998, the same decline in territorial males as time passed, seen in 1997, was clearly observed. Any trend in marked female or hatchling numbers is not as clear. A slight decrease in marked females can be noted and is in agreement with the observed death of a number of females during the observational period. Hatchlings also appeared to decline in number during the same short period, and by observation at least a few were known to be dead.

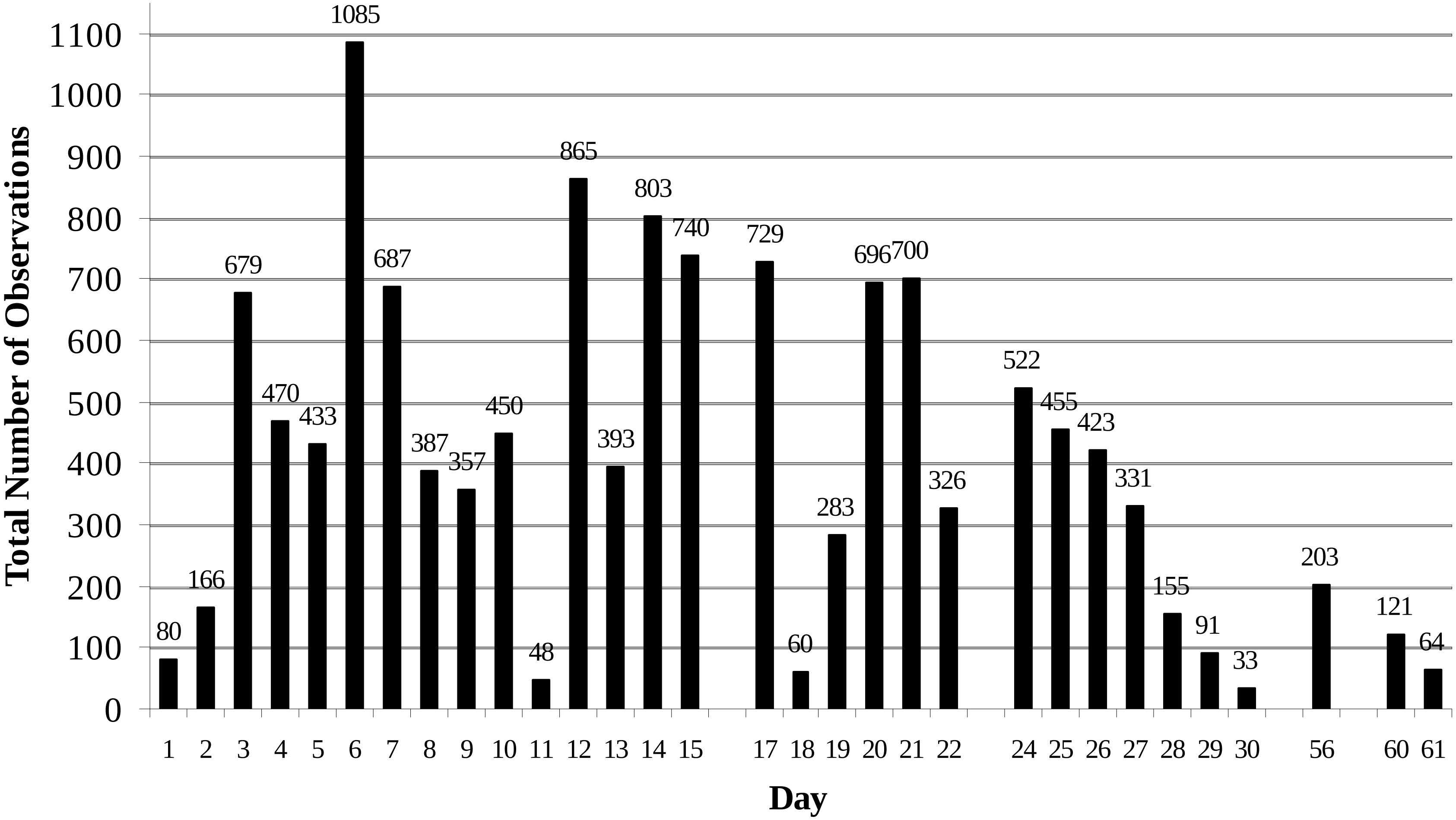


Figure 8. Total number of daily observations during the 1997 reproductive season.

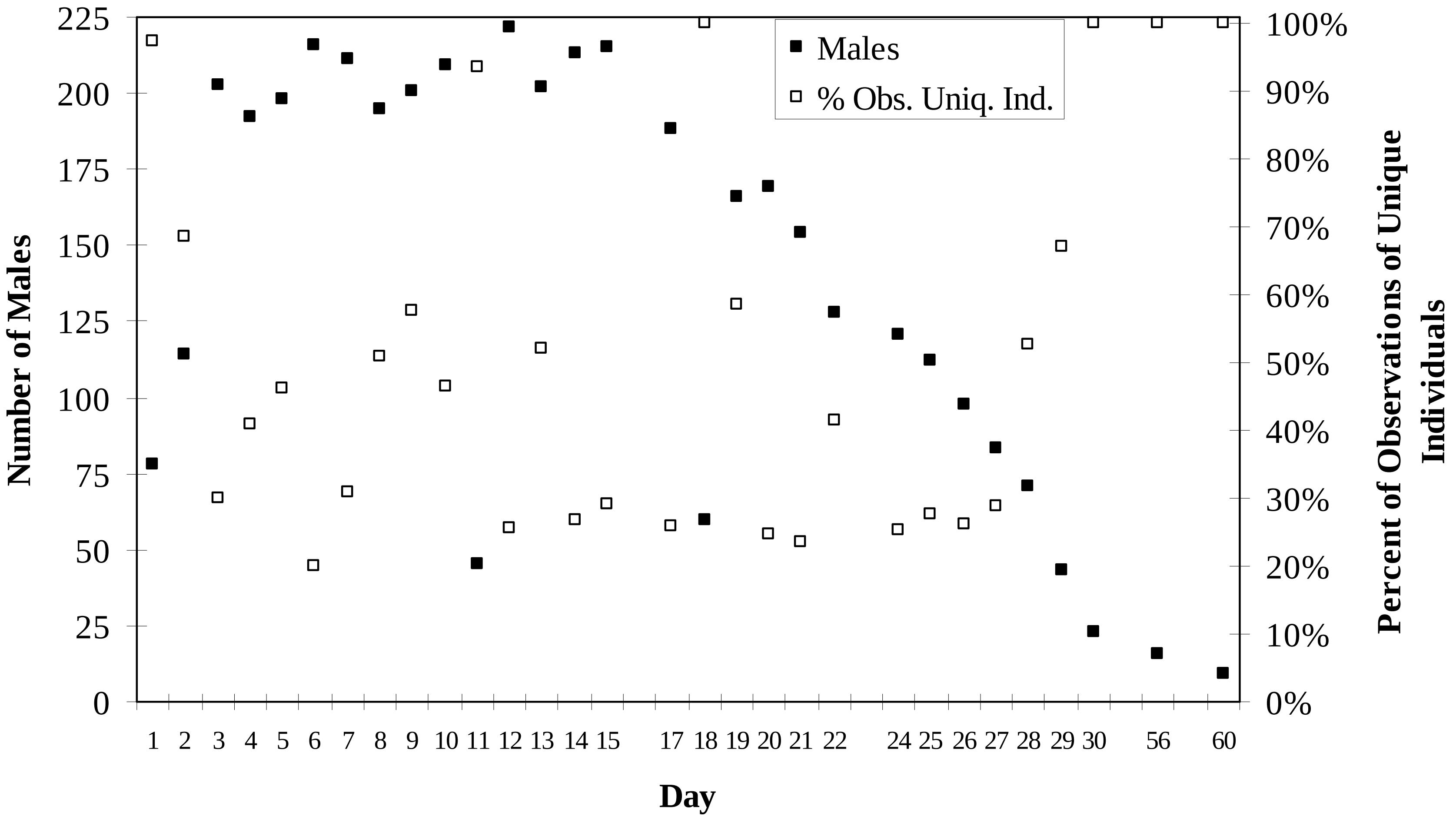


Figure 9. Daily total number of unique territorial males and percent of all territorial male observations that were of unique individuals during the 1997 reproductive season.

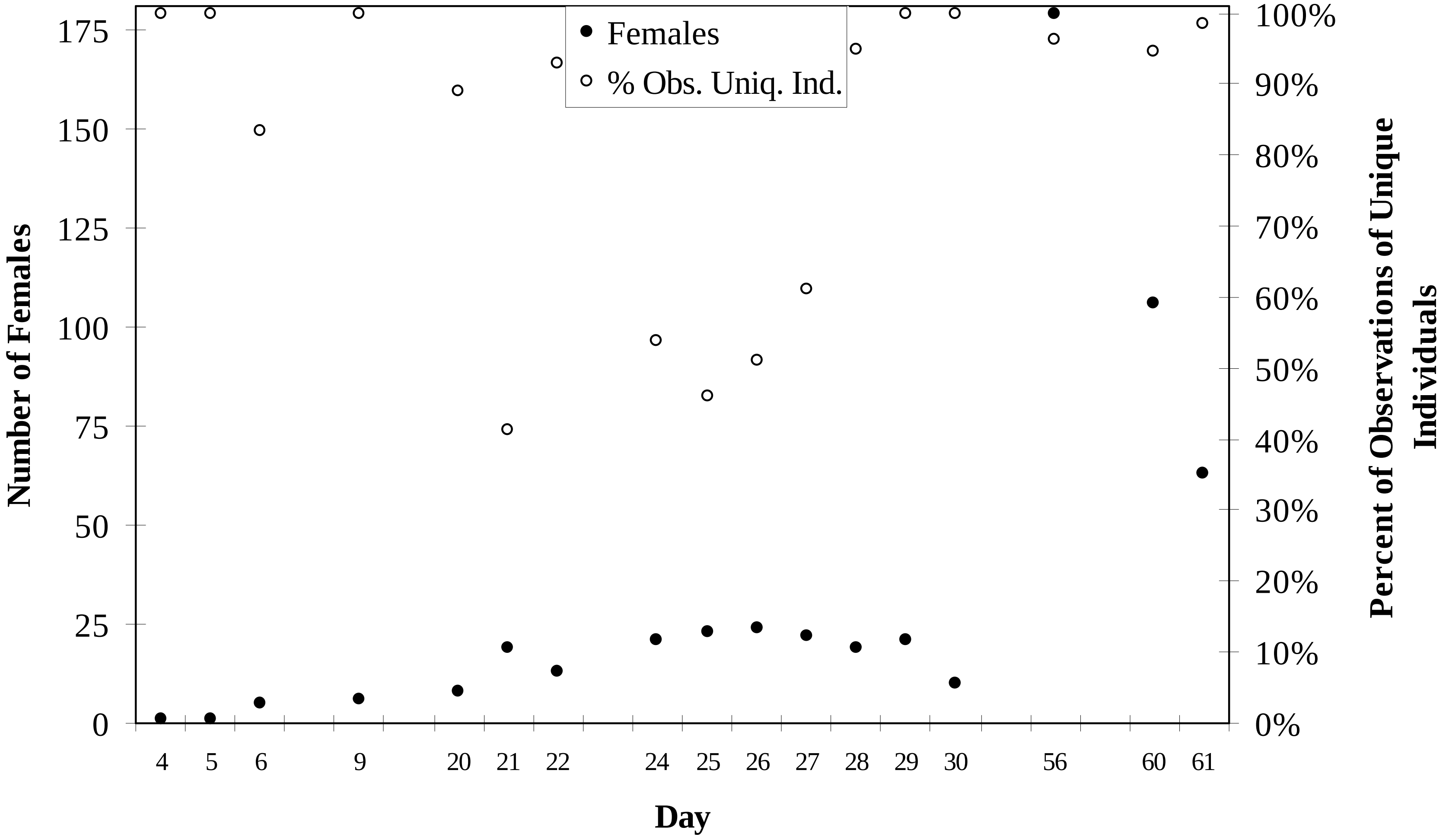


Figure 10. Daily total number of unique females and percent of all female observations that were of unique individuals during the 1997 reproductive season.

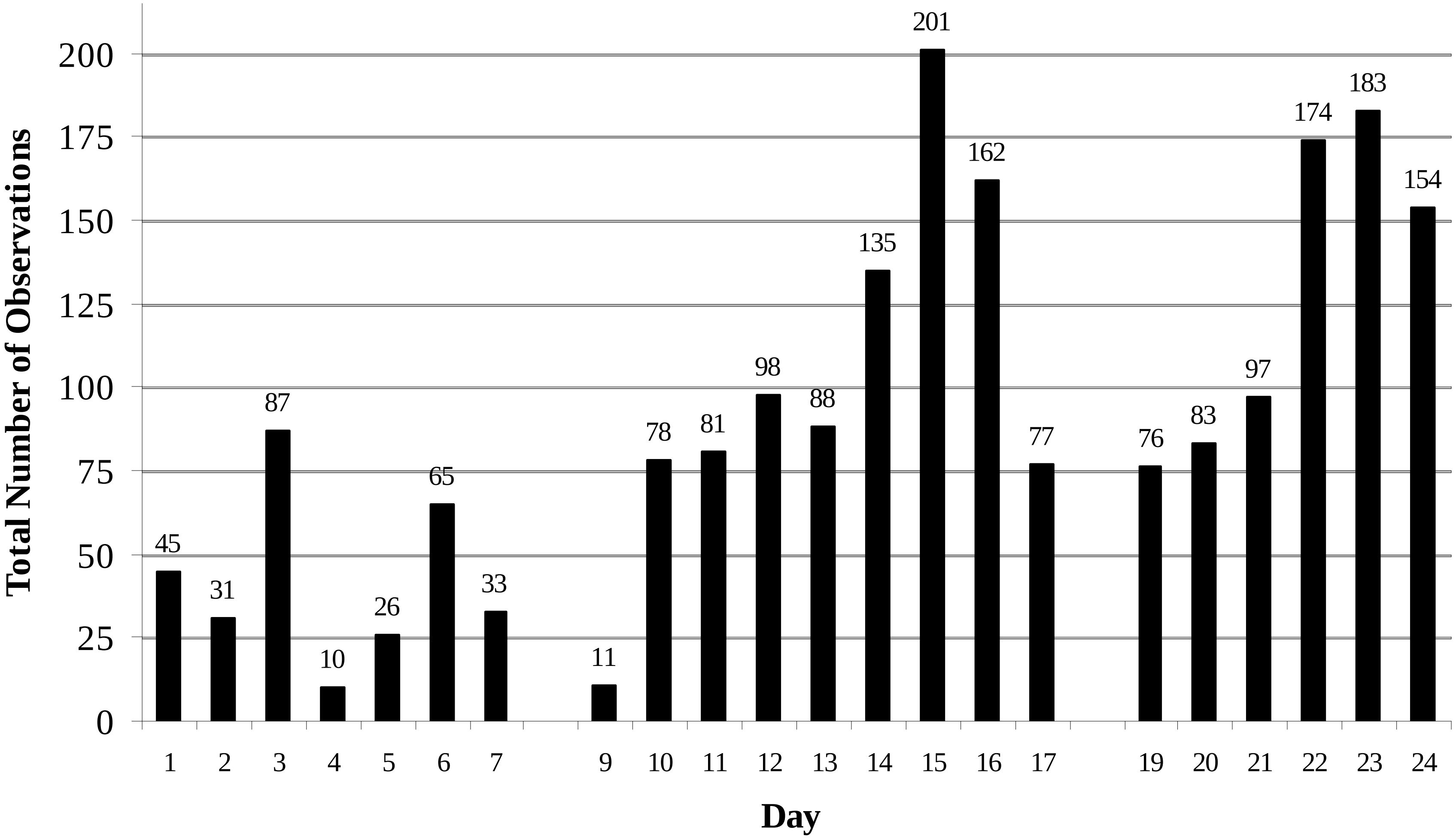


Figure 11. Total number of daily observations during May-June, 1997.

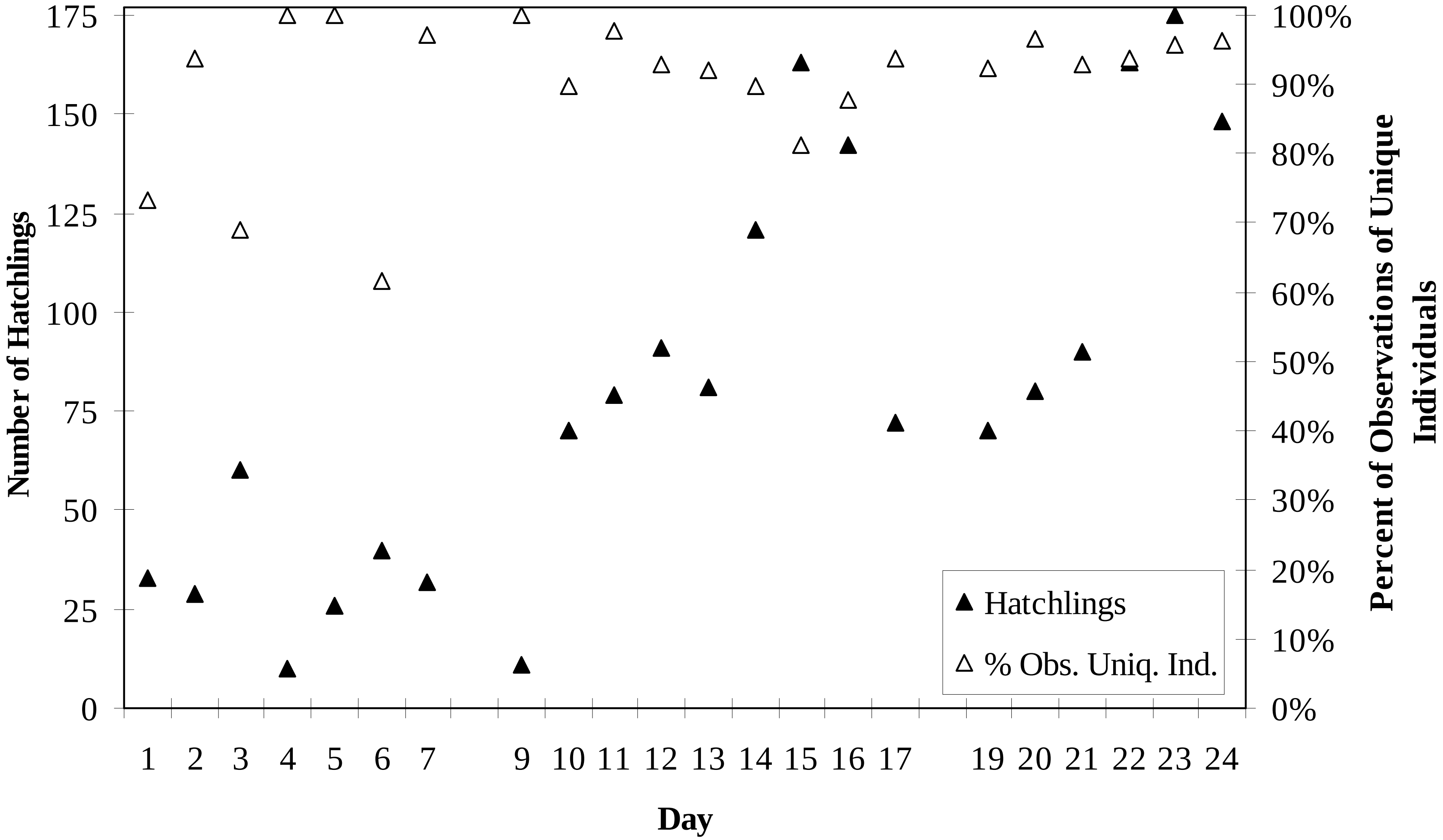


Figure 12. Daily total number of unique hatchlings and percent of all hatchling observations that were of unique individuals immediately following hatching in 1997.

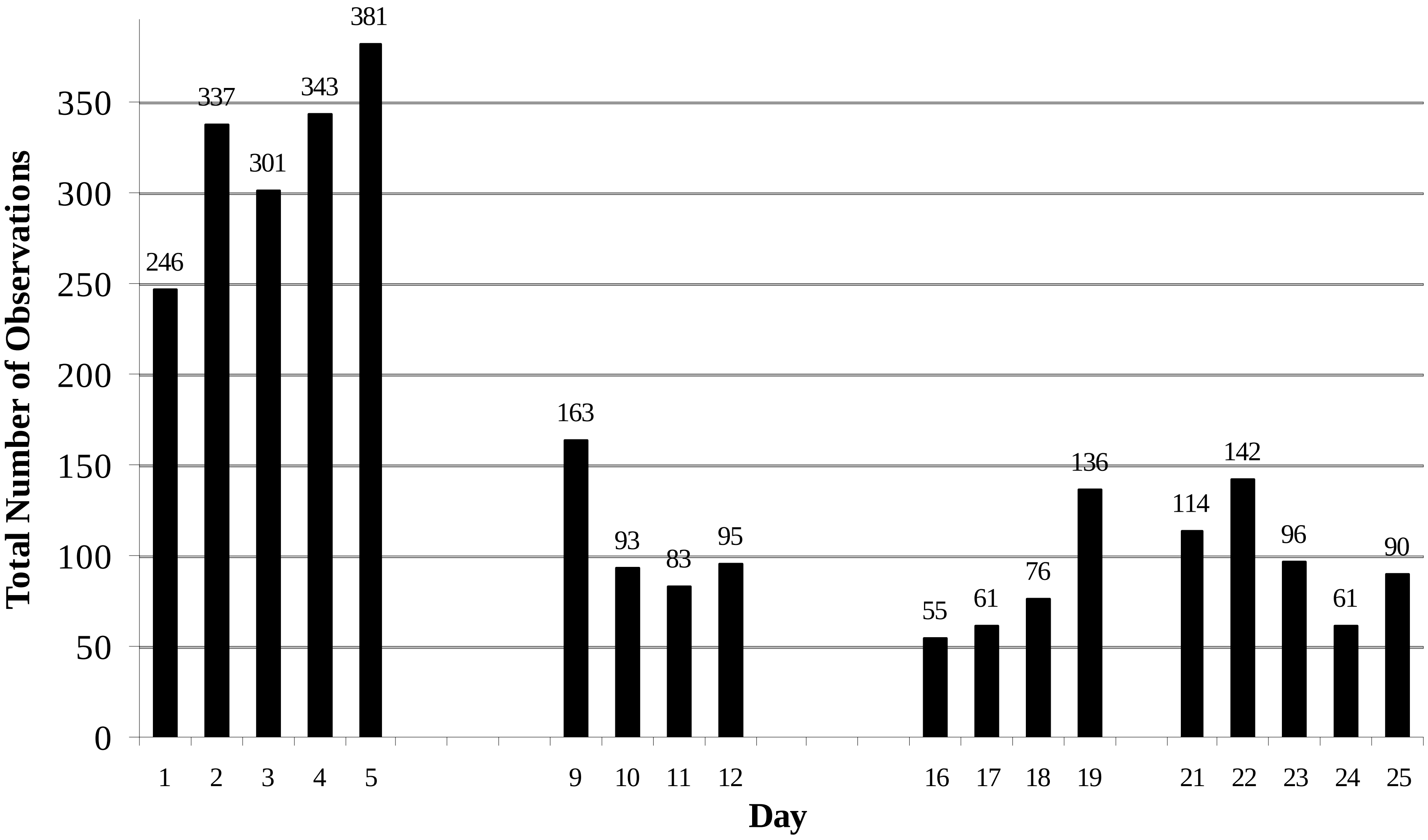


Figure 13. Total number of daily observations during the 1998 reproductive season.

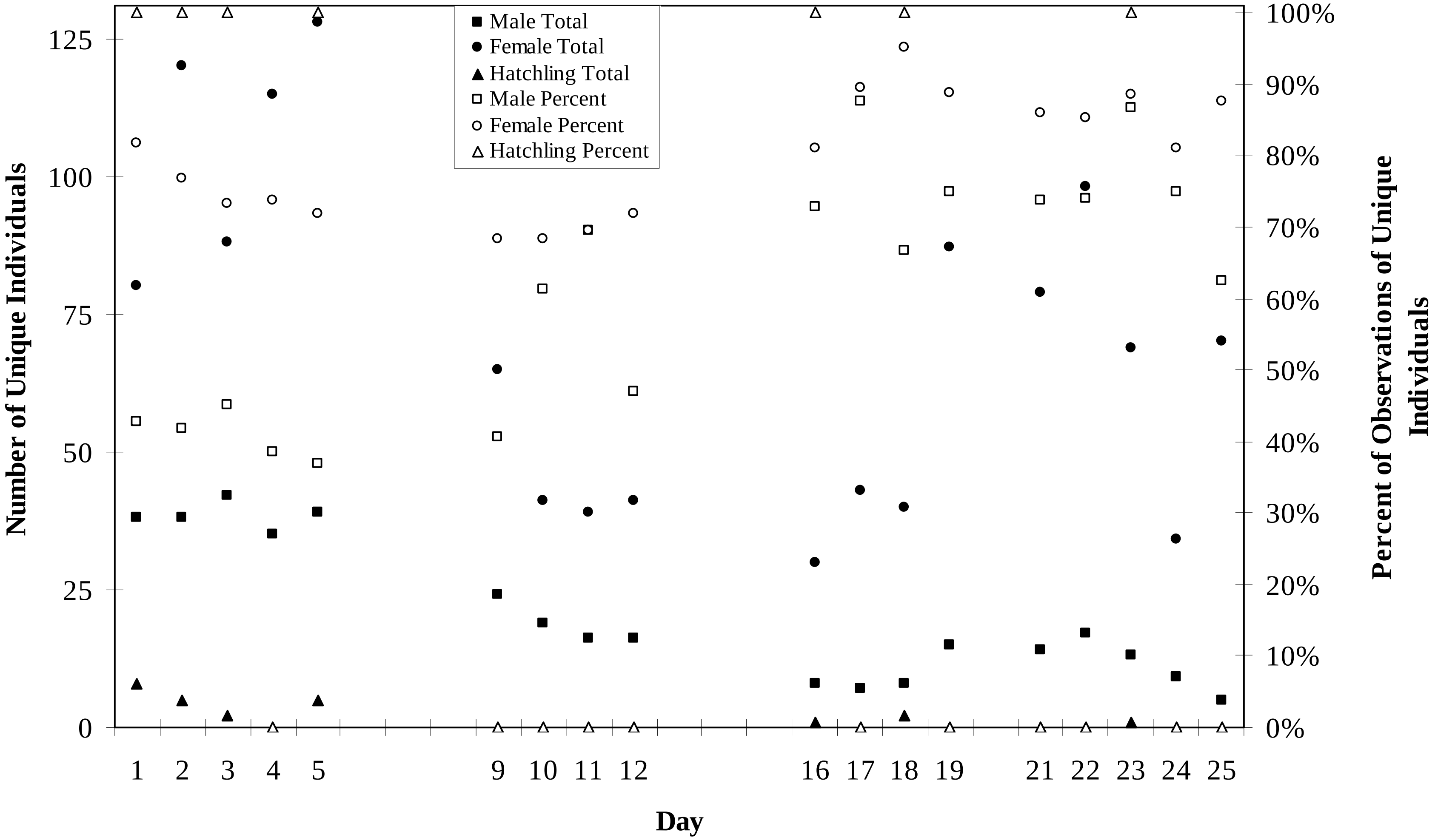


Figure 14. Daily total number of unique males, females, and hatchlings and percent of all male, female, and hatchling observations that were of unique individuals during the 1998 reproductive season.

Density Patterns

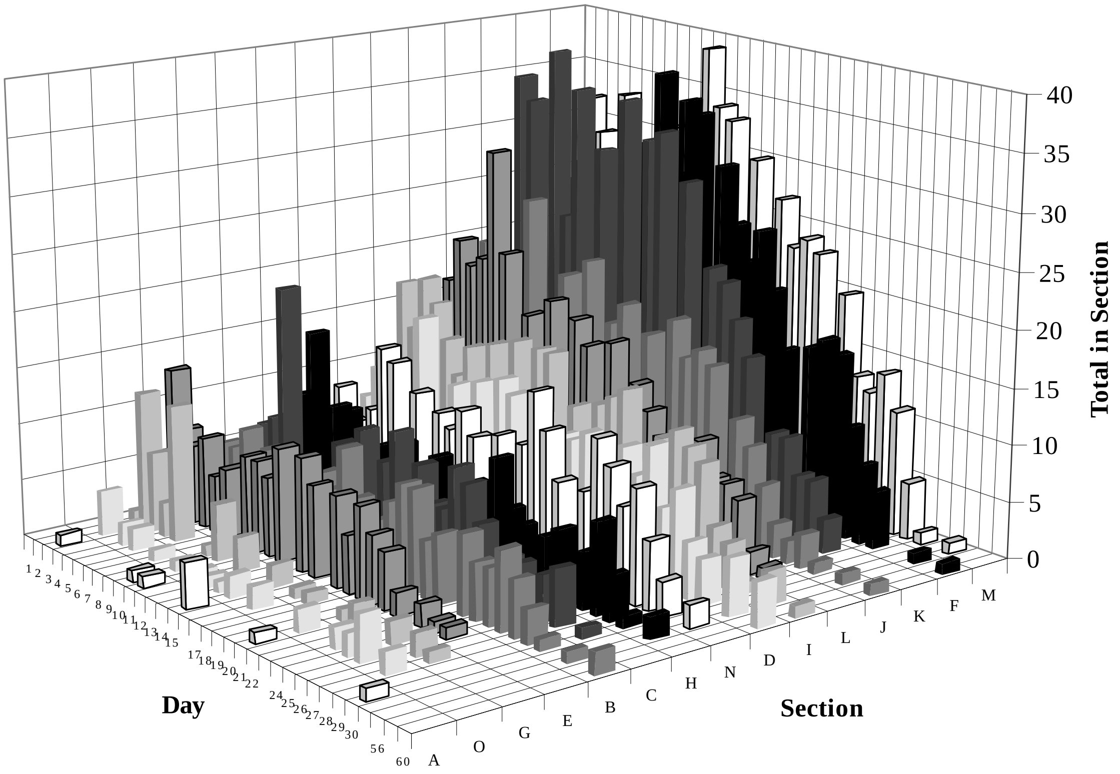
Changes in population density within marked sections of the islet are provided in the following 11 figures (Figure 15 - Figure 25). Territorial male, female, and hatchling data are provided. For particularly complex 3-D figures, an additional diagram containing daily information for individual sections as small bar charts on a map of the islet is provided.

Clear trends are visible documenting territorial male departure from the islet in both 1997 (Figure 15 and Figure 16) and 1998 (Figure 19 and Figure 20). During both years, the three sections with the highest number of males observed were the same (sections F, K, and M). Departure as time passed was evident both years, but is most clearly seen in the 1997 data.

Since females (in 1997) were initially captured and marked only if they had been observed copulating, relatively few were marked during the most active portion of the reproductive season. Later, as nests were dug, large numbers of females were captured, measured, and marked. Figure 17 indicates this graphically. The period between day 30 and day 56 was consumed by marking activities. Similar information is shown in Figure 18 for hatchlings captured later in 1997 (only without the large gap in time between marking and later observation). Few hatchlings were observed in 1998, so Figure 25 is rather sparse.

Relatively few males returned to the islet and set up territories during the 1998 reproductive season. Tendency toward departure later in the season is really only seen in the most densely populated sections, F, K, and M (see Figure 19 or Figure 20). Females observed in 1998 may have accumulated in or returned to areas previously occupied by territorial males that had departed as the season progressed. A relatively consistent increase in female density observed in sections F, M, K, and possibly others is striking (Figure 21 and Figure 22). Although some other factor cannot be ruled out, as number of observed males and females between days 19 and 23 increased (see also Figure 14).

Ratio of females to males (shown in Figure 23 and Figure 24) provides yet another view of how males left as time passed and (along with the prior two figures) how numbers of females possibly increased in the areas once occupied by territorial males. Reference to Figure 13 is helpful in evaluating whether simply more observations led to more females being reported in sections. While total number of daily observations was higher on some later days the increase is often not large enough to account for the greater abundance of females in some sections. For example, see days 23-25 in section F (Figure 21 or Figure 22) compared with total daily observations for days 23-25 (Figure 13) compared with the total daily observations of the previous few days. While the observation is not definitive, it is interesting.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
□ A			1							1	1				4						1									1				
░ O			4		2	2		1		1		1	1	1	2		2				2			2	2	4		2						
▒ G		1	12	7	3	12			1	5		3			2		1	1			1	2			2		2	1						
▨ E		13	8	7	8	5	6		8	8	7	10		10	8		8	5	8	6	5	2		2	1	1								
▓ B		3	6	6	8	6	7			5	4	7	6	10	6		5	7	9	9	5	6		7	5	5	7	5	3	1		1		2
■ C		5	7	8	20	3	4			8	2	10		8	11		9	6	6	10	9	6		4	4	1	4	5		1				
■ H		3	9	15		9	9	1	5	7		8	6	7	8		5	7	4	10	6	5		5	6	3	5	8	4	1		2		
□ N			9	6	4	8	14	13		11		10	9	11	9		10		10	15	12	8		8	13	11	8	10	6	3		2		
░ D		7	10	11		8	16			11	5	12		13	12		11	9	10	10	11	11		11	9	12	7	9	5	4		5		4
▒ I		17	13	18	16	13	10	13	6	14		15		15	15		11		12	13	14	8		11	12	11	10	5	4	1		3		1
▨ L		6	17	21	19	20	30	21		16		18	12	17	15		16		13	11	9	8		10	7	7	6	2	1					
▓ J		11	20	2	10	17	25	13	15	19		21		16	18		16		18	15	16	15		11	9	6	3	2	3	1		1		1
■ K		11	35	33	14	38	23	35		30		35	14	32	33		29		22	21	18	15		9	9	6	6	3						
■ F	1	6	17	25	27	18	28		25	30	5	37	28	35	34		30	25	22	25	20	15		16	17	16	10	7	5			1		1
□ M		1	32	29	24	33	27	28		31		34	39	34	33		30		27	23	24	23		20	13	12	14	11	5	1		1		

Figure 15. Territorial male density during the 1997 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for females in 1997. Totals indicate number of unique males observed within each section during a given day.

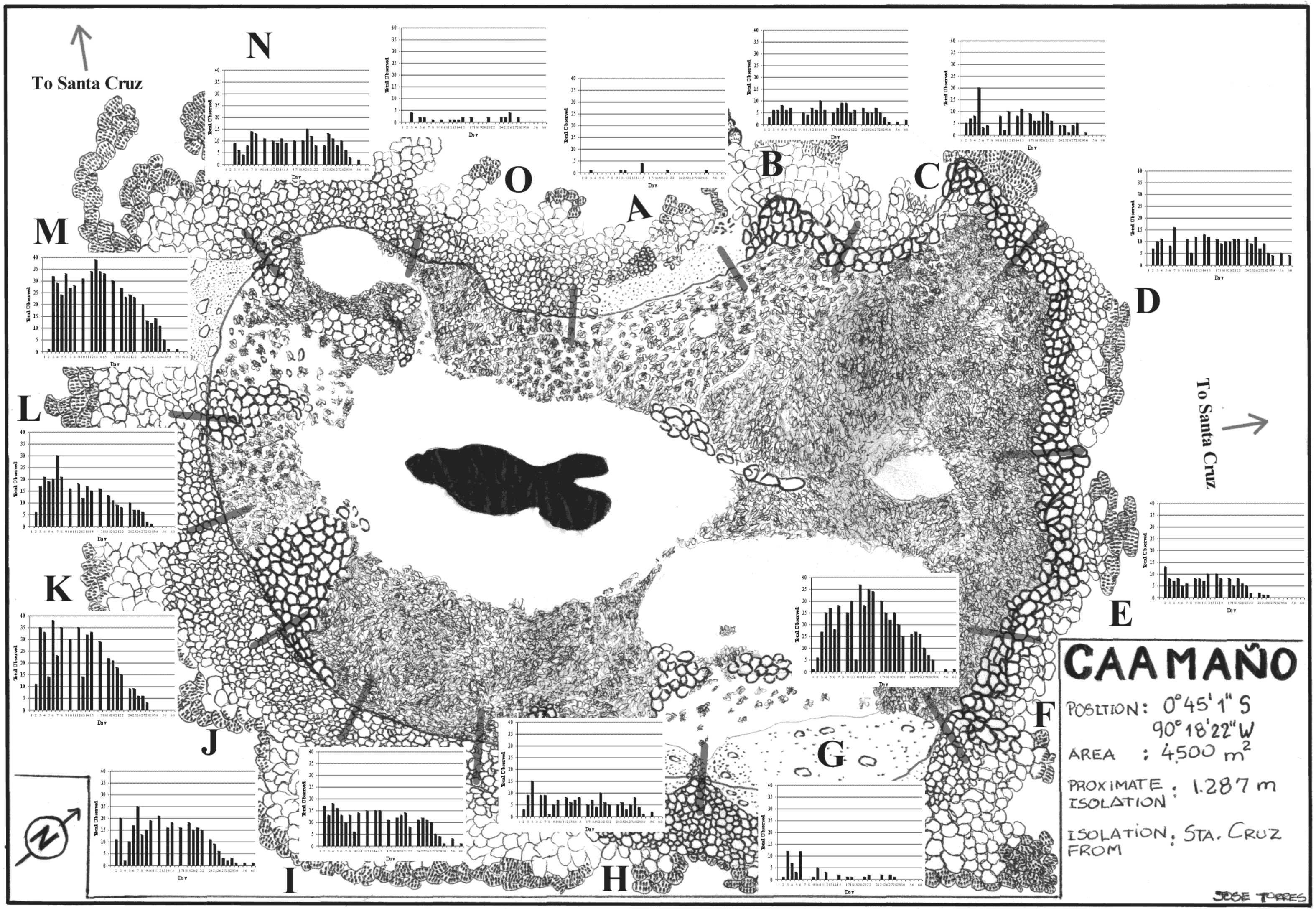
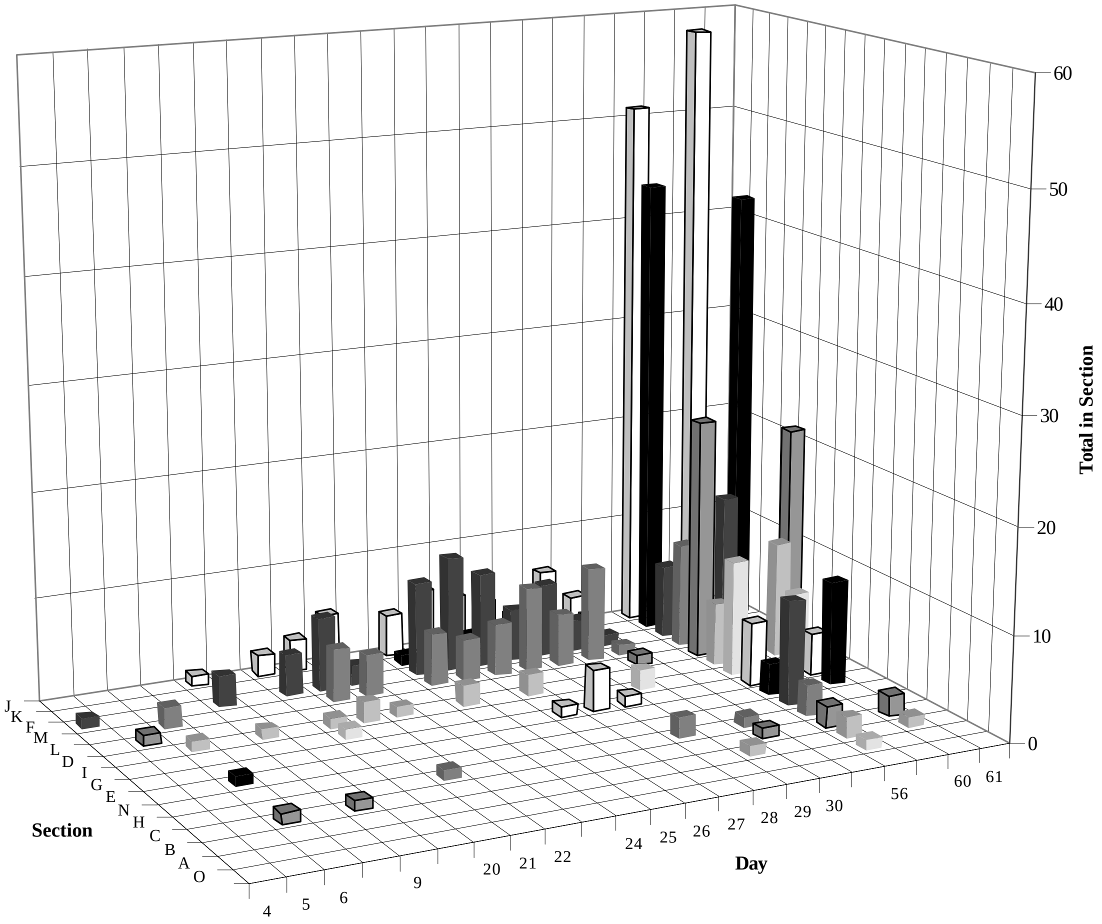
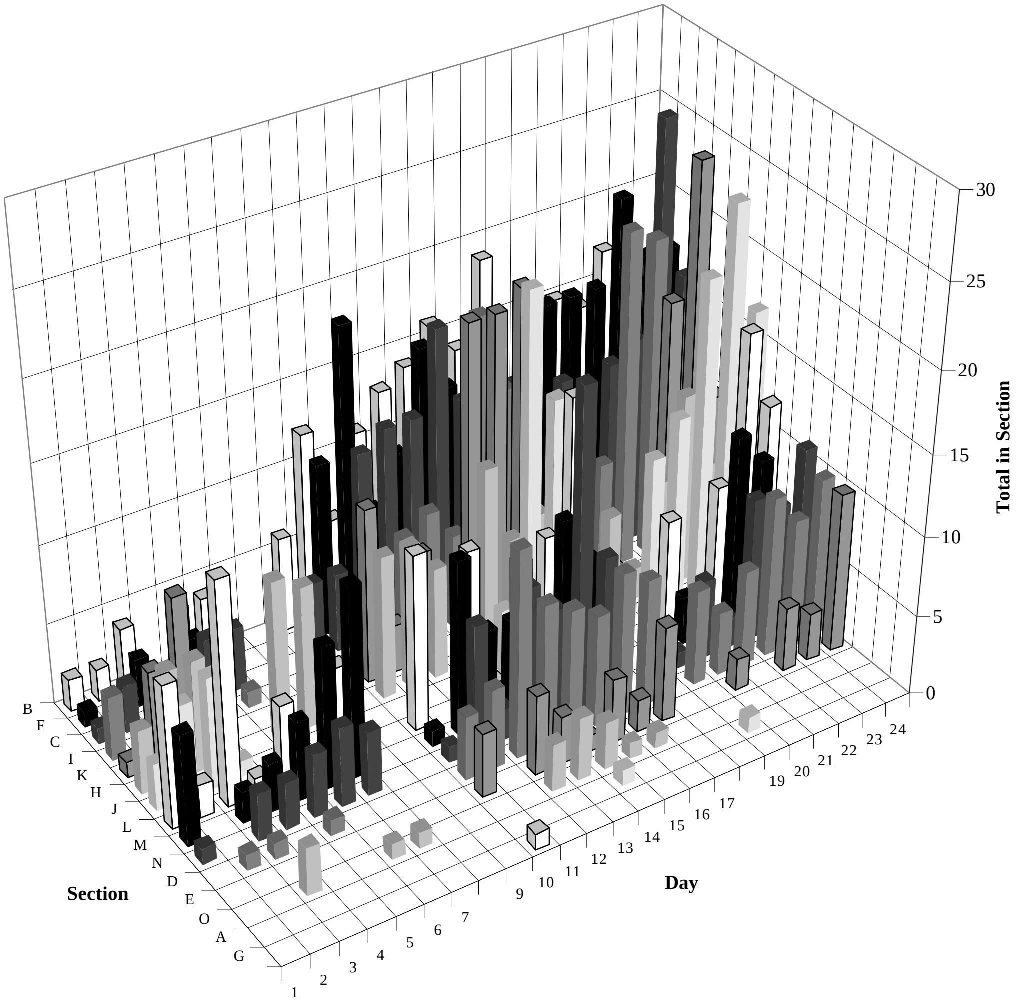


Figure 16. Map of territorial male density within sections (A-O) during the 1997 reproductive season.



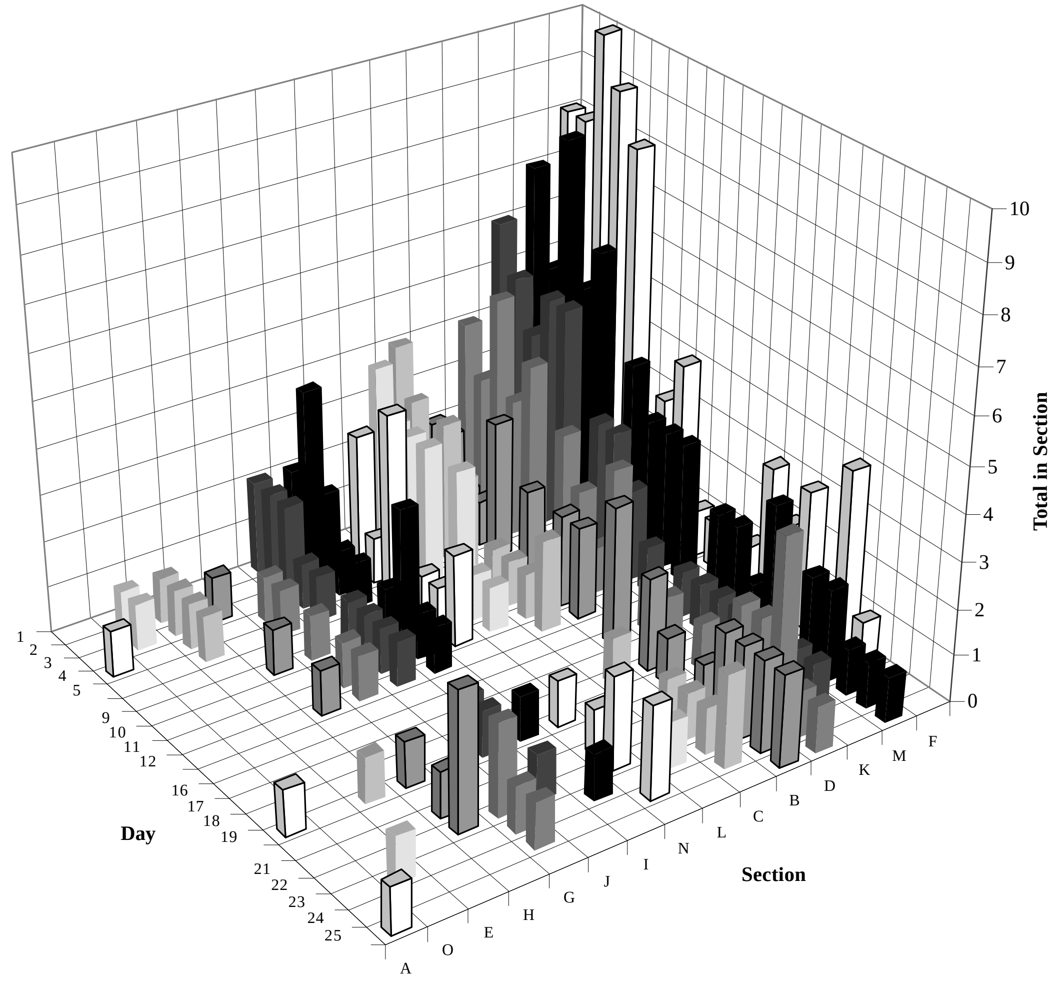
	4	5	6		9		20	21	22		24	25	26	27	28	29	30		56		60	61
□ O																						
■ A																			1			
■ B																1			2		1	
■ C			1		1												1		2		2	
■ H								1							2		1		3			
■ N																			10			
■ E			1																3		10	
□ G													1	4	1				6		4	
■ I							1									2			11		7	
■ D			1		1		1	2	1		2		2						6		11	
■ L		1															1		23			21
■ M			2					5	4		5	4	5	8	5	9	1		10			
■ F	1				3		4	7	2		9	11	9	5	7	3	1		7		13	
■ K									1		1	2	2	1	2		2		44			42
□ J					1		2	3	5		4	6	5	4	2	6	3		51		58	

Figure 17. Female density during the 1997 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for males in 1997. Totals indicate number of unique females observed within each section during a given day.



	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
☐ G										1														
■ A														1					1					
■ O			3			1	1					3	4	3	1	1								
■ E										4		5	3	1	4	2	6			2		4	3	10
■ D		1	1		1					4	5	13	9	8	7	9	8		6	4	6	10	8	10
■ N	1		3	3	4	5	4			1	8	2	9	7	20	9			1	5	2	9	8	11
■ M	7		2	3	5	9	12			1	11	6	6	3	11	8				3		12	10	4
☐ L	9	2	14	1	5	1	6			11		10	1	8	9	17				7		8	17	12
■ J	3	6	7	1										4	23	16			1	10	12	20	24	17
■ H	4	7	7			10	9			9	9	7	5	12	7	8	6		6	2	7	12	7	11
■ K	1	6	10	2						11	3	7		20	20	21				1		17	25	10
■ I	4	1	3			1					6	7	8	6	19	14	15		11	7	21	20	15	15
■ C	1	3	3		4	4			5	5	12	13	13	18	13	13	13		12	8	12	13	26	16
■ F	1	1	3		3	5				11	19	7	10	16	13	10	5		16	16	16	21	17	17
☐ B	2	2	4		4	4			6	12	6	11	13	14	16	14	19		16	15	14	17	15	15

Figure 18. Hatchling density within sections immediately following hatching in 1997. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations. Totals indicate number of unique hatchlings observed within each section during a given day.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ A				1											1						1
▨ O		1	1																1		
▨ E		1	1	1	1										1						
▨ H			1				1			1					1		1	3			
▨ G				1	1		1		1	1								2	1	1	
▨ J	2	2	2	1	1		1	1	1	1				1	1			1			
■ I	2	4	2	1	1		1	3	1	1					1				1		
□ N			3	1	4		1	1	2						1		1	2		2	
▨ L	2	4	3	3	3		3	1	1										1		
▨ C	4	3	1	3	2		1	1	1	2				1			1	1	1	2	
▨ B	2	2	1	1	3		2		2	2		3		2	1		1	2	2	2	2
▨ D	4	3	5	3	4		3	2	1	3		1	1		1		2	2	4	1	1
▨ K	6	5	4	5	5		3	3	2	1		1	1	1	1		1	1	1		
■ M	7	5	8	5	6		4	3	3	3		2	2	1	3		2	2	1	1	1
□ F	8	8	10	9	8		3	4	1	1		1	3	2	3		4	1			

Figure 19. Territorial male density during the 1998 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for females in 1998. Totals indicate number of unique males observed within each section during a given day.

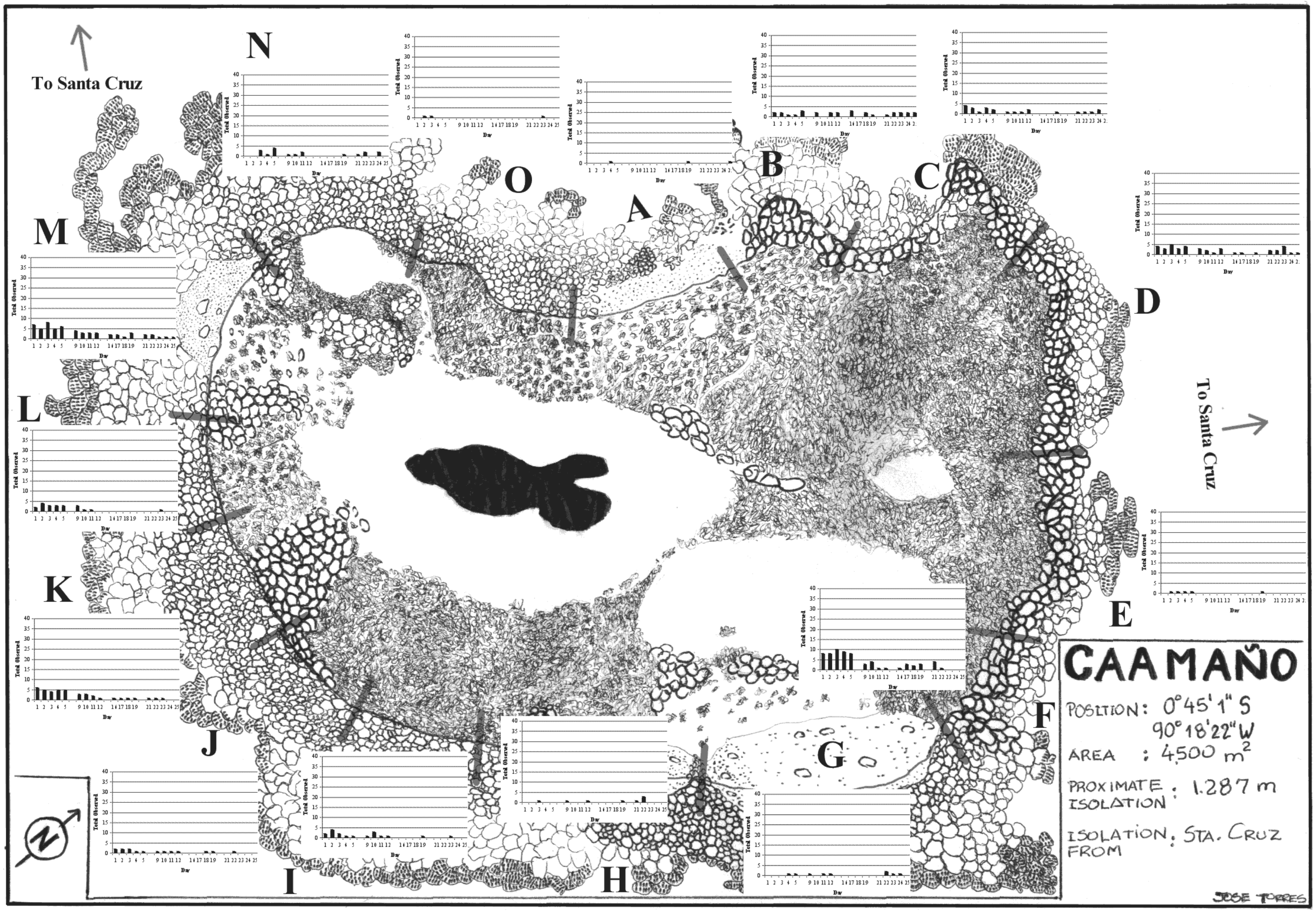
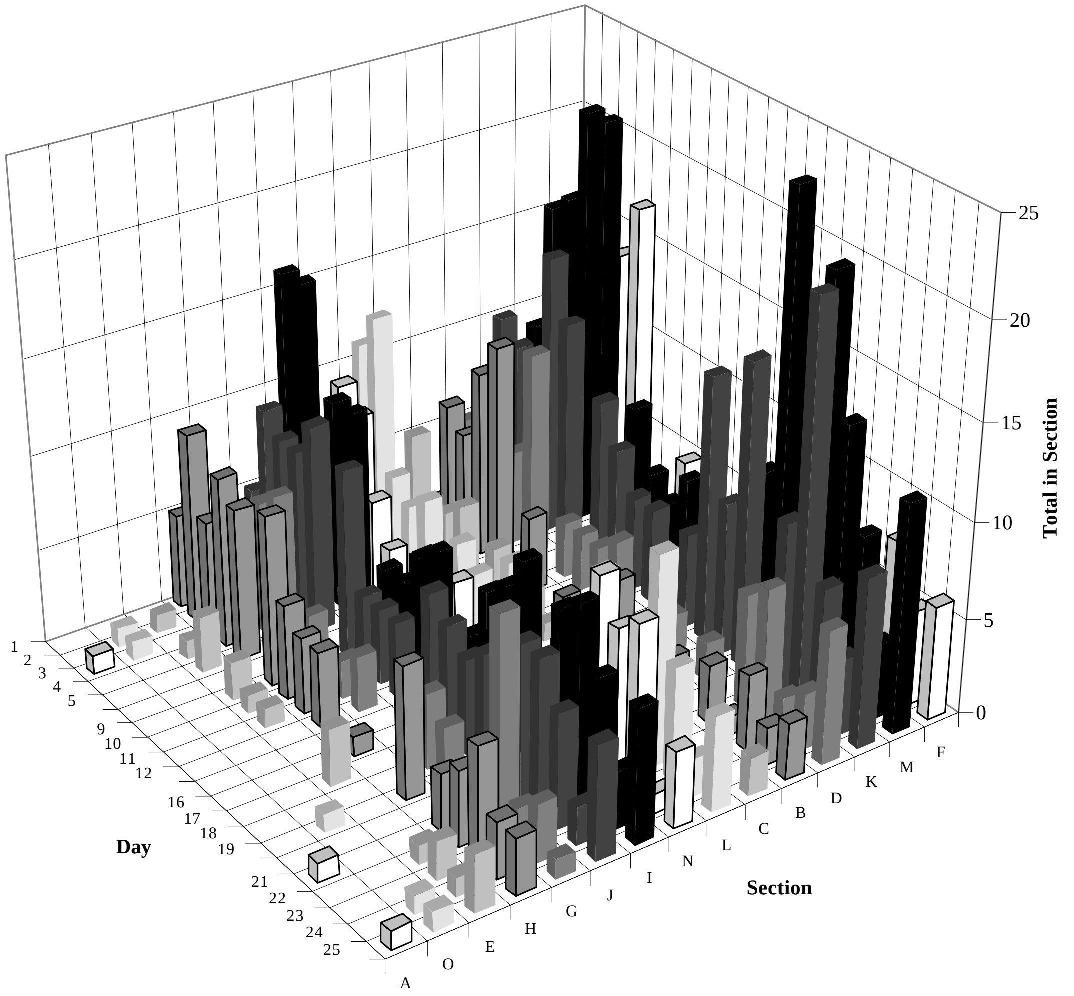


Figure 20. Map of territorial male density within sections (A-O) during the 1998 reproductive season.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
☐ A			1														1				1
■ O		1	1												1					1	1
■ E		1		1	3		2	1	1				3					1	2	1	3
■ H	5	10	6	9	8		9	5	4	4		1			7		3	4	6	3	3
■ G	4		4	7	8		3		2	3			1	4	3		1	11	2	3	1
■ J	5	10	9	9	11		10	4	4	4		7	6	5	6		8	8	6	2	6
■ I	16	16	9	11	11		4	4	6	7		4	7	8	10		9	10	7	3	7
☐ N	2	10	9	5	3		4	3	4	1		1	1	1	5		10	8	9	1	4
■ L	11	13	5	4	5		4	3	2	5		1	3	2	8		5	11	6	2	5
■ C	3	6	2	3	4		3	1	2	1		1									2
■ B	1	7	6	10	12		4		1	1		4			2		3	1	4	2	3
■ D	5	8	3	5	11		3	3	3	4			2		2		6	7	2	3	7
■ K	10	9	8	15	12		9	7	5	5		5	14	8	16		9	21	7	4	9
■ M	9	16	17	22	22		8	5	4	6		4	4	9	24		21	14	9	4	12
☐ F	8	13	8	14	17		2	5	1			2	2	3	3		3	2	8	5	6

Figure 21. Female density during the 1998 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for males in 1998. Totals indicate number of unique females observed within each section during a given day.

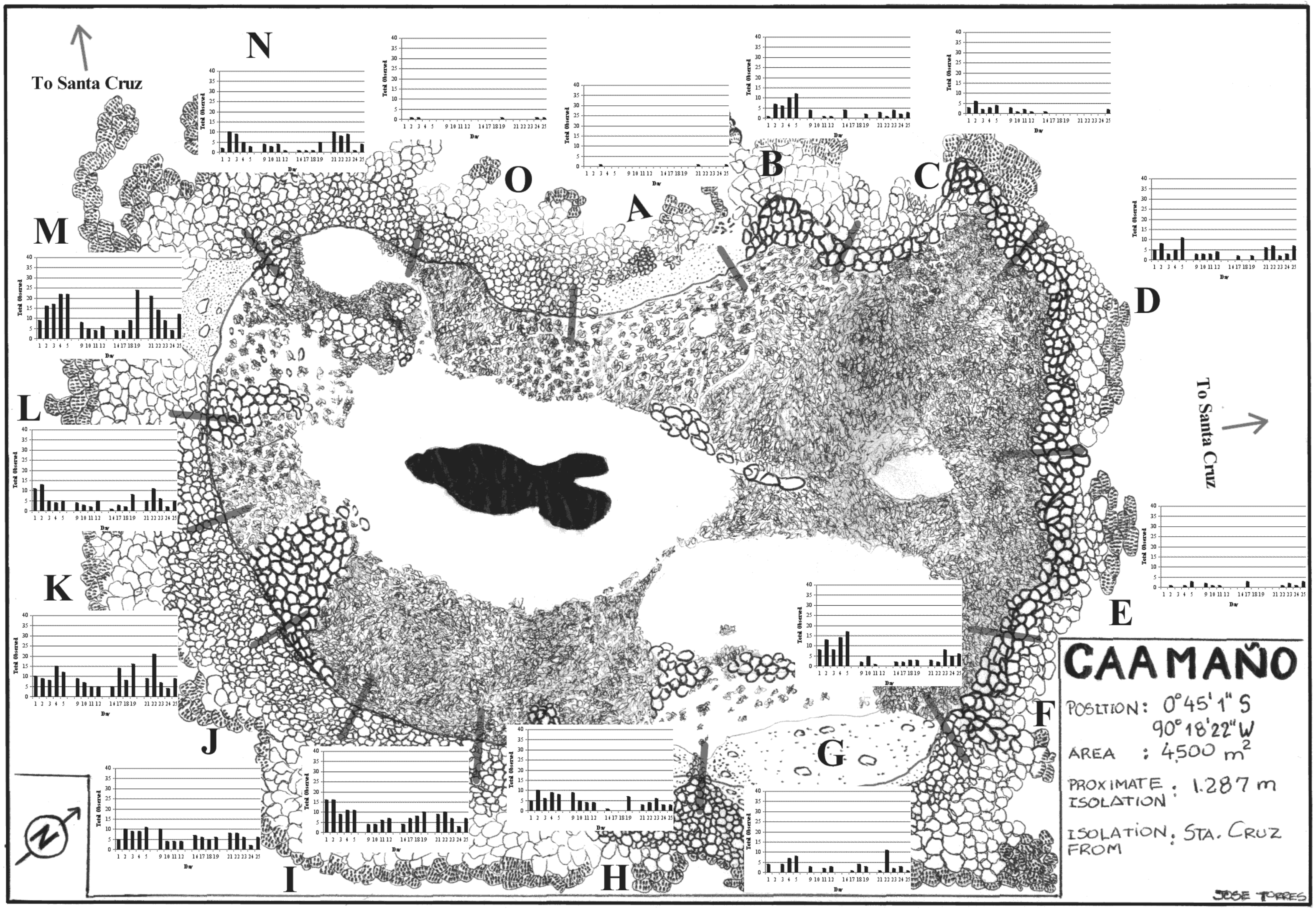
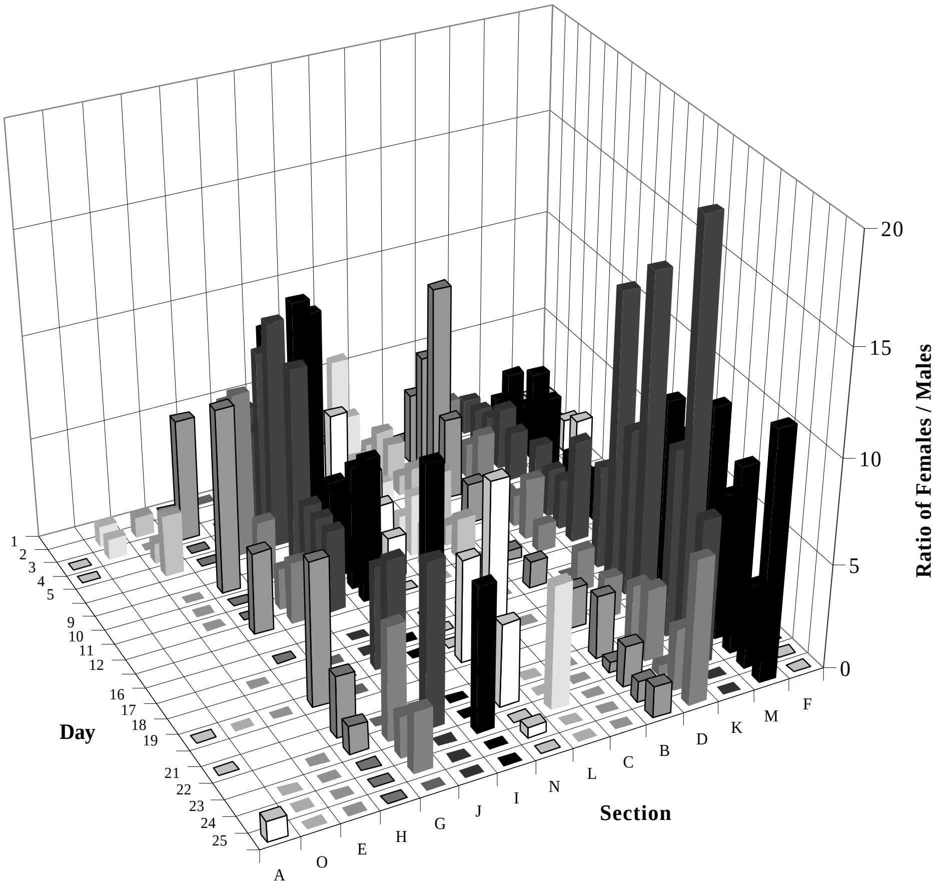


Figure 22. Map of female density within sections (A-O) during the 1998 reproductive season.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
☐ A			0.0	0.0											0.0		0.0				1.0
▨ O		1.0	1.0												0.0				0.0	0.0	0.0
▨ E		1.0	0.0	1.0	3.0		0.0	0.0	0.0				0.0		0.0			0.0	0.0	0.0	0.0
▨ H	0.0	0.0	6.0	0.0	0.0		9.0	0.0	0.0	4.0		0.0			7.0		3.0	1.3	0.0	0.0	0.0
▨ G	0.0		0.0	7.0	8.0		3.0		2.0	3.0			0.0	0.0	0.0		0.0	5.5	2.0	3.0	0.0
▨ J	2.5	5.0	4.5	9.0	11.0		10.0	4.0	4.0	4.0		0.0	0.0	5.0	6.0		0.0	8.0	0.0	0.0	0.0
■ I	8.0	4.0	4.5	11.0	11.0		4.0	1.3	6.0	7.0		0.0	0.0	0.0	10.0		0.0	0.0	7.0	0.0	0.0
☐ N	0.0	0.0	3.0	5.0	0.8		4.0	3.0	2.0	0.0		0.0	0.0	0.0	5.0		10.0	4.0	0.0	0.5	0.0
▨ L	5.5	3.3	1.7	1.3	1.7		1.3	3.0	2.0	0.0		0.0	0.0	0.0	0.0		0.0	0.0	6.0	0.0	0.0
▨ C	0.8	2.0	2.0	1.0	2.0		3.0	1.0	2.0	0.5		0.0		0.0			0.0	0.0	0.0	0.0	0.0
▨ B	0.5	3.5	6.0	10.0	4.0		2.0		0.5	0.5		1.3		0.0	2.0		3.0	0.5	2.0	1.0	1.5
▨ D	1.3	2.7	0.6	1.7	2.8		1.0	1.5	3.0	1.3		0.0	2.0		2.0		3.0	3.5	0.5	3.0	7.0
▨ K	1.7	1.8	2.0	3.0	2.4		3.0	2.3	2.5	5.0		5.0	14.0	8.0	16.0		9.0	21.0	7.0	0.0	0.0
■ M	1.3	3.2	2.1	4.4	3.7		2.0	1.7	1.3	2.0		2.0	2.0	9.0	8.0		10.5	7.0	9.0	4.0	12.0
☐ F	1.0	1.6	0.8	1.6	2.1		0.7	1.3	1.0	0.0		2.0	0.7	1.5	1.0		0.8	2.0	0.0	0.0	0.0

Figure 23. Ratio of total number of unique females to total number of unique territorial males observed within sections during the 1998 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for males and females in 1998. Zero indicates there were either females and no males or males and no females. Numbers in table are rounded off.

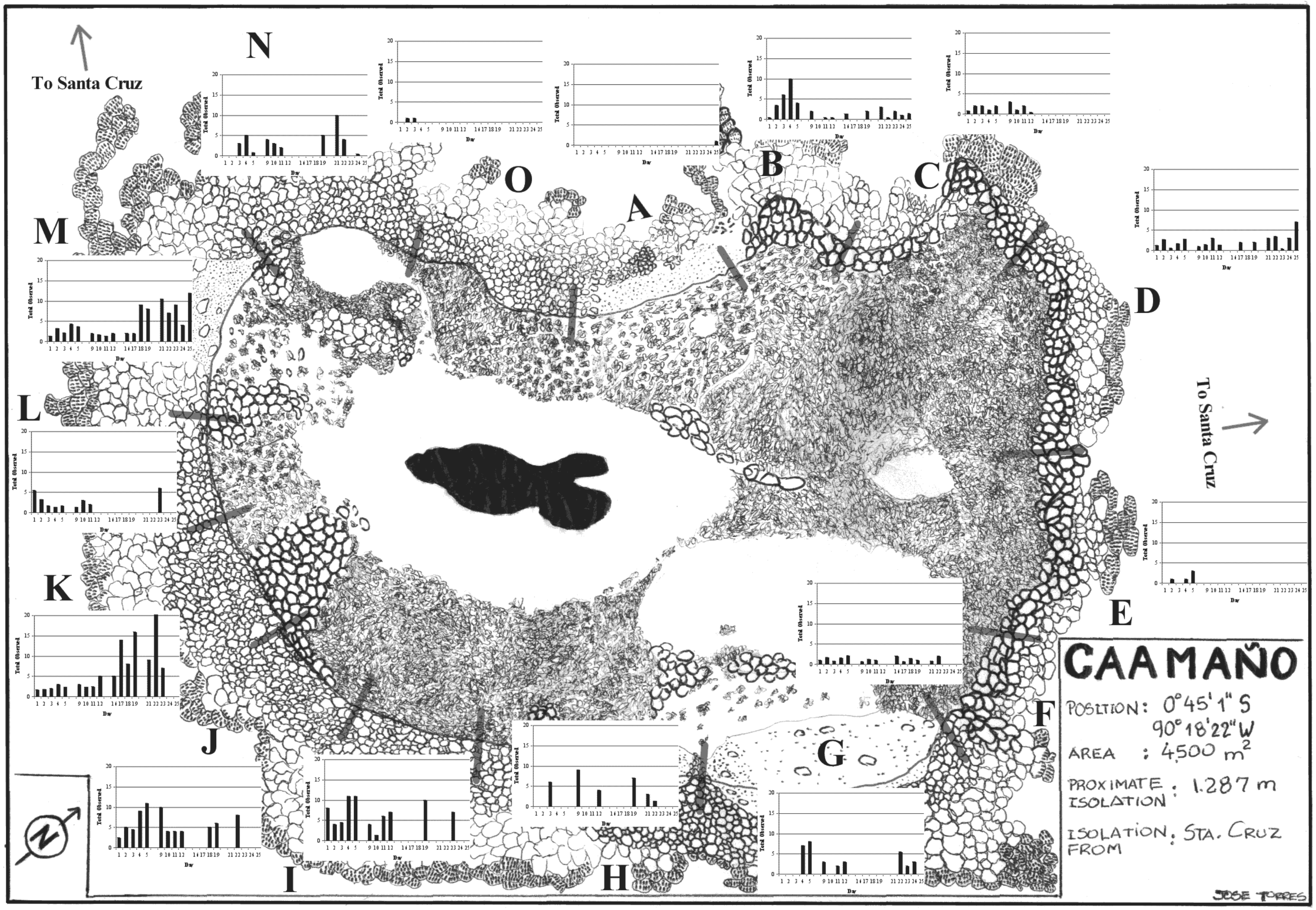
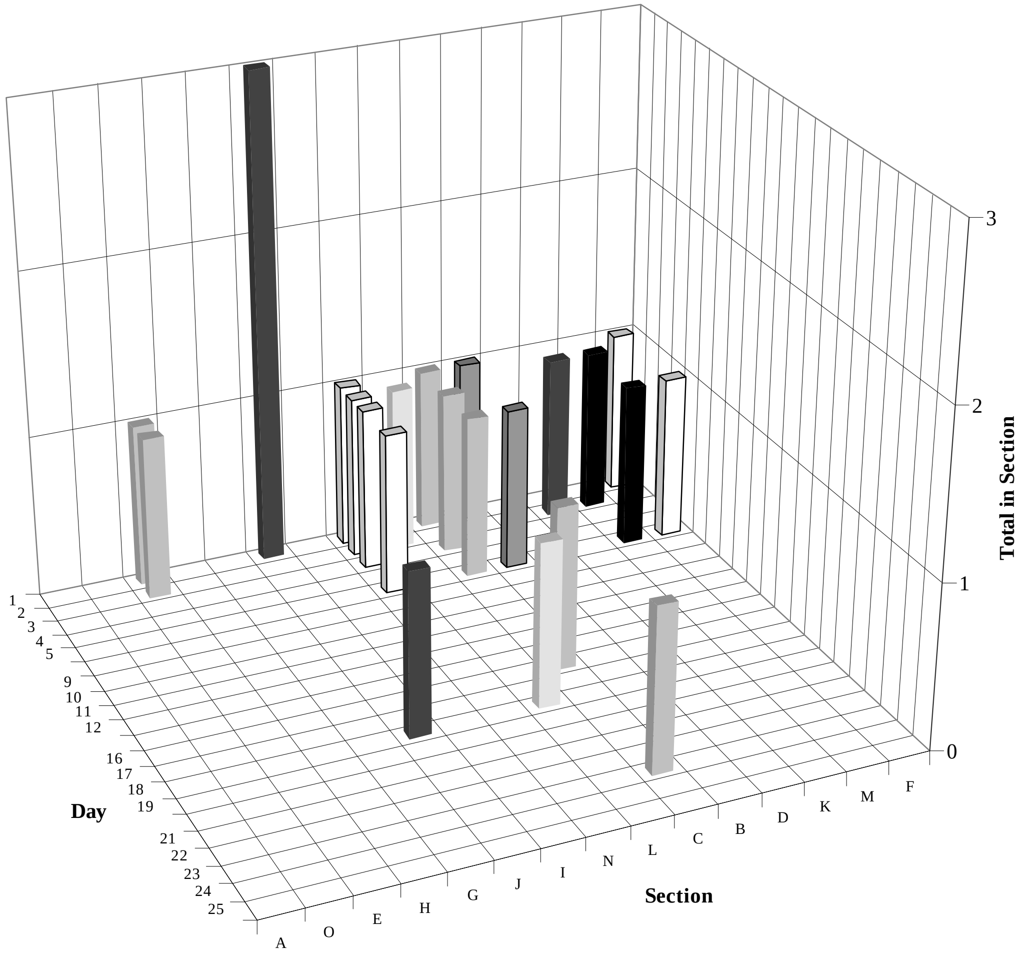


Figure 24. Map of female to male density ratio within sections (A-O) during the 1998 reproductive season.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ A																					
■ O																					
■ E	1	1																			
■ H																					
■ G																					
■ J	3													1							
■ I																					
□ N	1	1	1		1																
■ L		1												1							
■ C	1		1		1							1							1		
■ B	1				1																
■ D																					
■ K		1																			
■ M		1			1																
□ F	1				1																

Figure 25. Hatchling density during the 1998 reproductive season. Sections (A-O) are identified in Figure 1. Day indicates sequential day since start of observations and corresponds with days listed for males and females in 1998. Totals indicate number of unique hatchlings observed within each section during a given day.

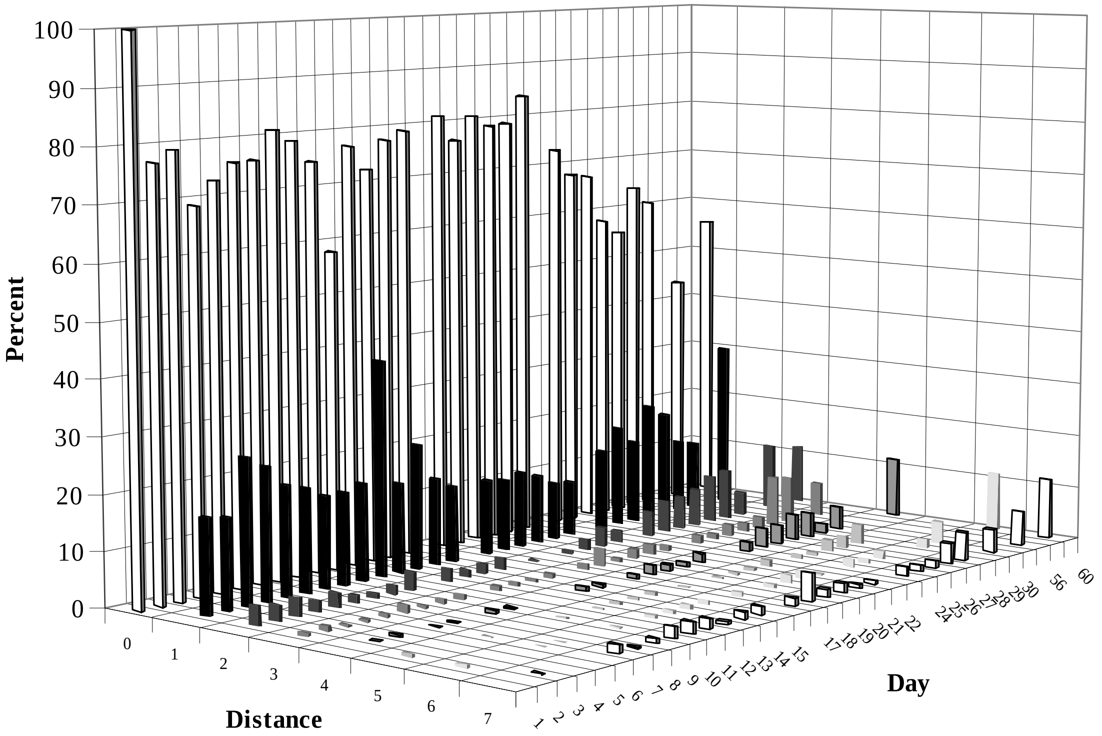
Movement Patterns

Changes in observed location relative to initial observation location are shown in the following five charts. Marked sections (see description and figure beginning on page 13) were recorded for all individuals at the time of observation. Section of first sighting was used as a baseline to calculate all further observed distances from the original. Since the sections were located in a circle, observation in a section on either side of the original was calculated as being one section from that of the original observation.

During the 1997 reproductive season, the charts for both territorial males (Figure 26) and marked females (Figure 27) indicate that, as time passed, higher proportions of individuals were observed two or more sections away from the section of original observation. For territorial males it is important to note that as this occurred there were fewer and fewer marked territorial males left on the islet (see Figure 9 on page 39). Also important to note for marked females is the fact that between days 30 and 56 a large amount of marking took place, so from day 56 on there were relatively high numbers of individuals being observed for the first time in an “original” location (see Figure 10 on page 40). Capture of females for marking took place as they entered the islet’s interior to dig nests and lay eggs, away from the perimeter that had marked sections used as reference points in daily observations. For this reason, the last three days of observations (56, 60, and 61) do not show a continuation of the trend of an increasing proportion of individuals being observed further and further away.

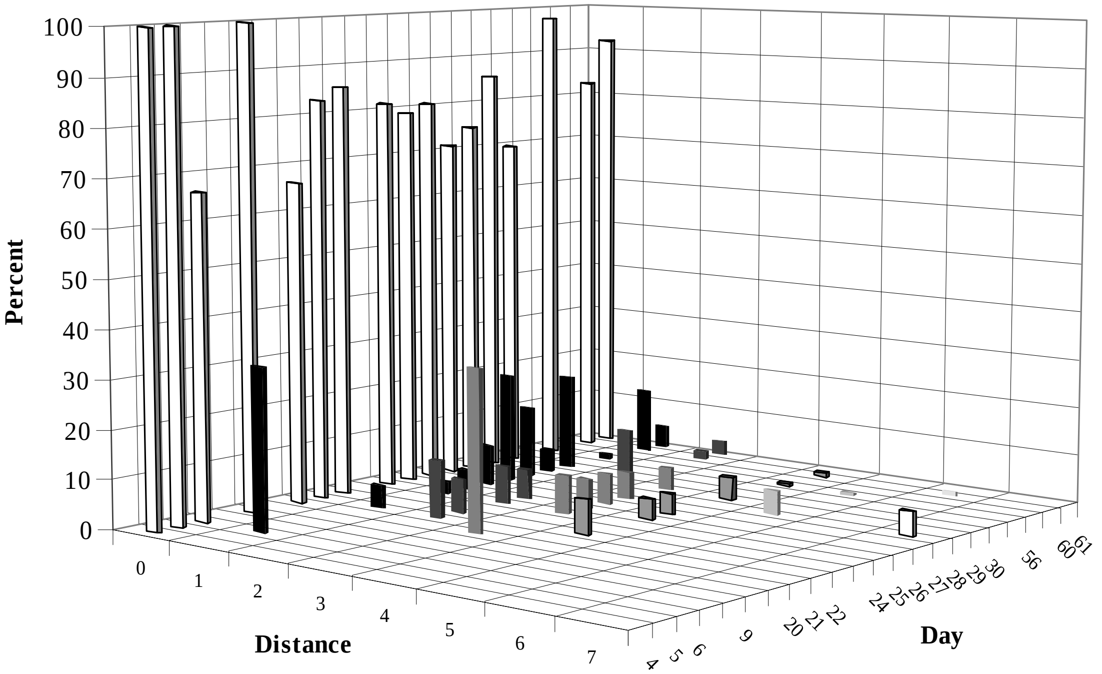
For hatchlings later in 1997, the trend appears to be the same as that observed in territorial males and marked females during the reproductive season. As time passed (see Figure 28) higher proportions of hatchlings were observed at greater distances from their original observation point. If not for continued marking of newly hatched individuals at the same time observations were taking place, the trend may have been even more pronounced.

In sharp contrast to the observations for territorial males and marked females presented for the 1997 reproductive year (see Figure 26 and Figure 27), no clear trend is observable for the same groups of marked individuals during the 1998 reproductive season (see Figure 29 and Figure 30). Both marked groups were found quite widely during the whole period of observation. Males did begin to wander a little farther and leave the islet near the end of observations, but changes in location were not nearly as pronounced as during the previous reproductive season. There were not enough hatchling observations to create a meaningful chart for the period of time covering the 1998 reproductive season.



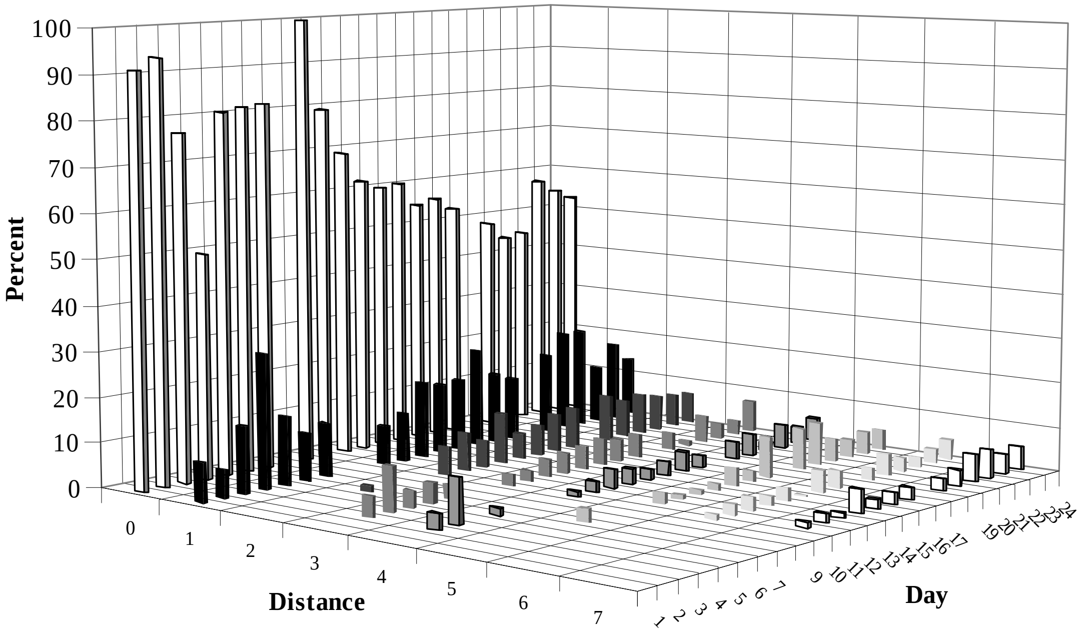
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
□ 7			0				1	0	1	2	2	2	1	1	1		1	5	1	2	1	1		2	1	1	4	5		4		6		11
■ 6		1				0	0			0		1	1	1	1		1		1	2	2	0		1	1	2			2	4				11
■ 5		1				0				0		0	1	1	1		0		0	0	1	1		1	0	2	2	4						
▨ 4			0	0		0	0		1	0				1	1		1	2	1	1	2			2	3	3	5	4	2	4				11
■ 3		1	1	0	0	1	1	1	1	1		1	1	1	1		1	3	1	2	2	1		1	1	2	2	2	9	9		6		
■ 2		4	3	3	2	3	1	1	2	3		2	2	2	2		0		1	2	4	2		5	6	6	7	9	9	4		13		11
■ 1		17	17	27	24	20	19	17	17	18	40	17	23	16	14		14	13	14	13	11	10		15	19	16	22	20	14	13		31		
□ 0	100	77	79	69	73	76	76	81	79	75	58	77	73	78	79		81	77	81	79	79	84		73	68	67	58	56	64	61		44		56

Figure 26. Territorial male movement patterns during the 1997 reproductive season. Distance is a measure (in sections, see Figure 1) of how far an individual was from its initial section of observation. Day indicates sequential day since start of observations and corresponds with days listed for females in 1997. Percent indicates daily the proportion of observed individuals seen at a given distance from their initial observation section. Numbers in table are rounded off.



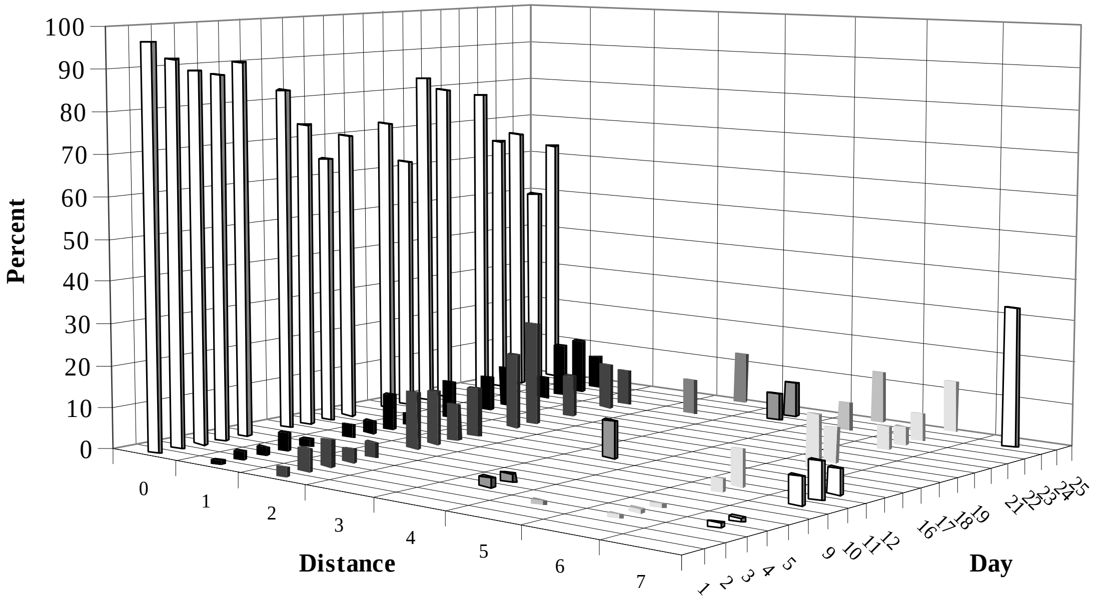
	4	5	6		9		20	21	22		24	25	26	27	28	29	30		56		60	61
□ 7															5							
░ 6																					1	
░ 5															5				1			
▨ 4									7			4	4			5			1		1	
▨ 3							33				8	6	6	6		5						
■ 2								12	7		8	6					10				2	3
■ 1			33					5			3	4	9	23	15	5	20		1		14	5
□ 0	100	100	67		100		67	83	86		82	79	81	71	75	86	70		98		83	92

Figure 27. Female movement patterns during the 1997 reproductive season. Distance is a measure (in sections, see Figure 1) of how far an individual was from its initial section of observation. Day indicates sequential day since start of observations and corresponds with days listed for males in 1997. Percent indicates daily the proportion of observed individuals seen at a given distance from their initial observation section. Numbers in table are rounded off.



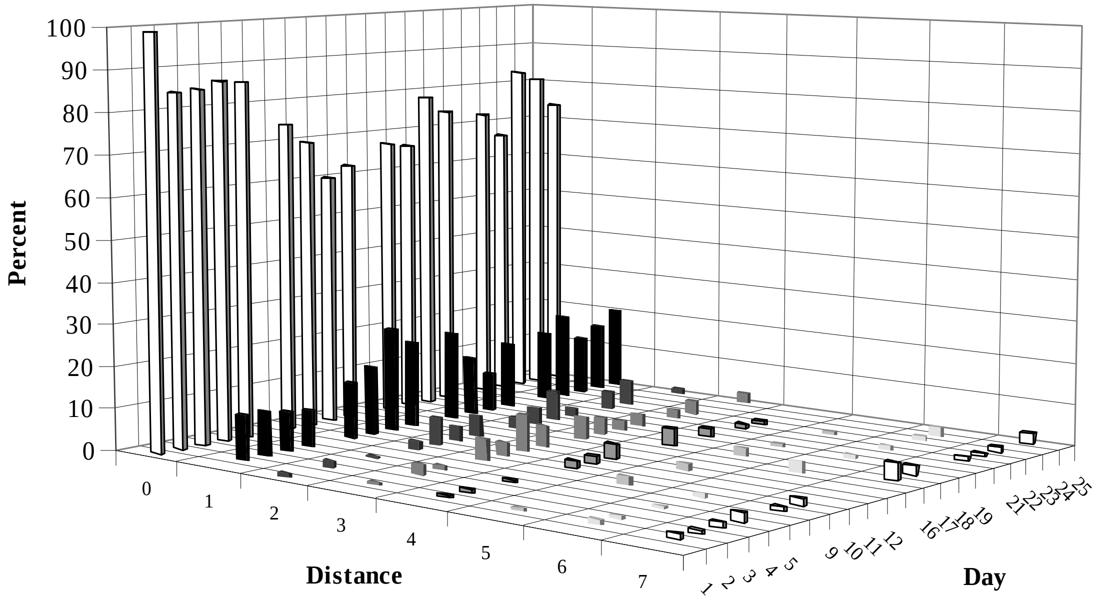
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
□ 7											1	2	1	5	2	2	3		3	4	6	6	4	5
■ 6										1	2	3	2	3	0	5	4		3	5	3	2	3	5
■ 5							3				2	1	1	1	4	2	9		9	10	5	4	5	5
■ 4			3	10		2				1	2	4	3	2	3	4	3		4	5	2	5	4	5
■ 3			5	10	4	5	3			3	2	4	5	5	6	5	5		4	1	6	3	3	7
■ 2						2				6	9	6	11	6	7	9	9		11	8	9	8	8	7
■ 1	9	6	15	30	15	11	12			9	11	17	16	16	22	16	14		18	23	23	13	18	14
□ 0	91	94	77	50	81	82	82		100	79	69	62	60	61	55	56	53		49	45	45	57	55	53

Figure 28. Hatchling movement patterns immediately following hatching in 1997. Distance is a measure (in sections, see Figure 1) of how far an individual was from its initial section of observation. Day indicates sequential day since start of observations. Percent indicates daily the proportion of observed individuals seen at a given distance from their initial observation section. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ 7				1	1			6	9	6										33	
▨ 6			1	1	1			3	9				13	8			6	4	7		13
▨ 5			1																7		13
▨ 4				2	2					9									7	8	
▨ 3																		9			13
▨ 2	2	5	6	3	4		14	13	9	12		18	25		10		11	9			
■ 1	1	2	2	4	2		3	3	9	3		9		8	10		6	13	13	8	
□ 0	97	92	89	88	91		83	74	65	71		73	63	83	80		78	65	67	50	63

Figure 29. Territorial male movement patterns during the 1998 reproductive season. Distance is a measure (in sections, see Figure 1) of how far an individual was from its initial section of observation. Day indicates sequential day since start of observations and corresponds with days listed for females in 1998. Percent indicates daily the proportion of observed individuals seen at a given distance from their initial observation section. Numbers in table are rounded off.

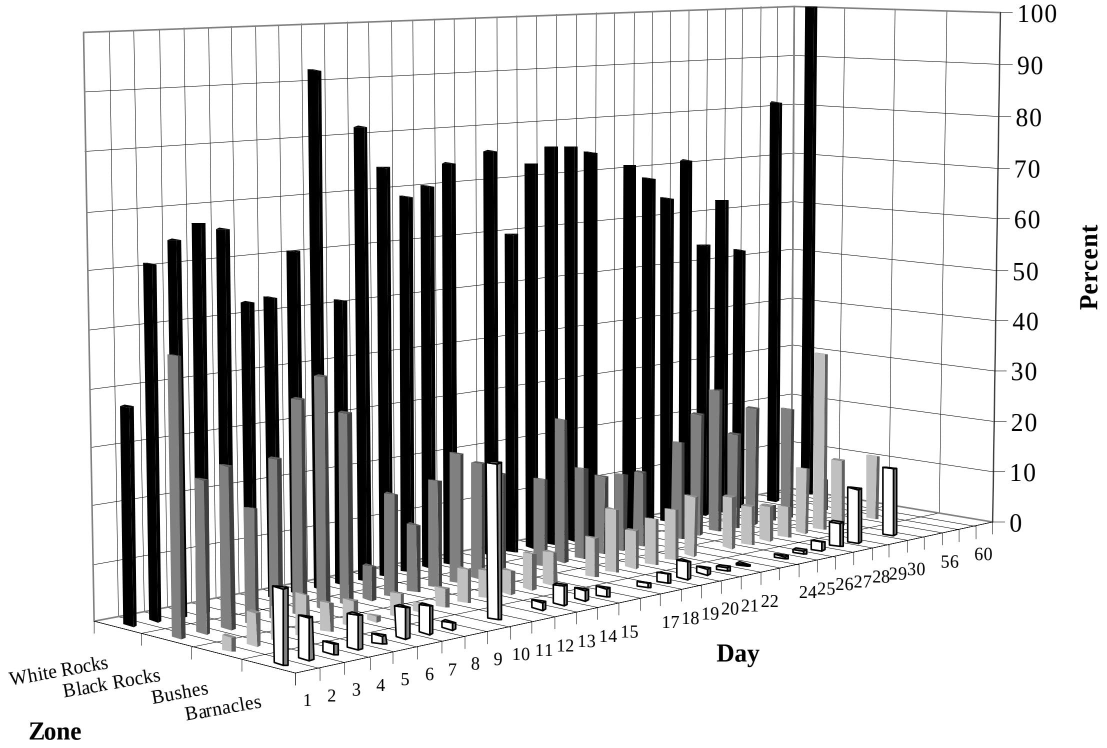


	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ 7		1	1	1	2		1	2					4	2			1	1	1		3
░ 6		1	1		1		1					3			1		1		1	2	
▒ 5		1					2			2			2		1			1			
▨ 4		1	1		1			2	2	4			4		2		1	1			
▓ 3		1		3	1		5	3	9	5		5	4	2	3		2	3			3
■ 2	1		2		1		2	7	4	5		3	4	7	2		4	6			1
■ 1		11	11	10	9		14	17	25	21		22	15	10	16		17	21	14	17	20
□ 0	99	85	85	87	86		75	70	61	63		68	67	79	74		73	67	83	81	74

Figure 30. Female movement patterns during the 1998 reproductive season. Distance is a measure (in sections, see Figure 1) of how far an individual was from its initial section of observation. Day indicates sequential day since start of observations and corresponds with days listed for males in 1998. Percent indicates daily the proportion of observed individuals seen at a given distance from their initial observation section. Numbers in table are rounded off.

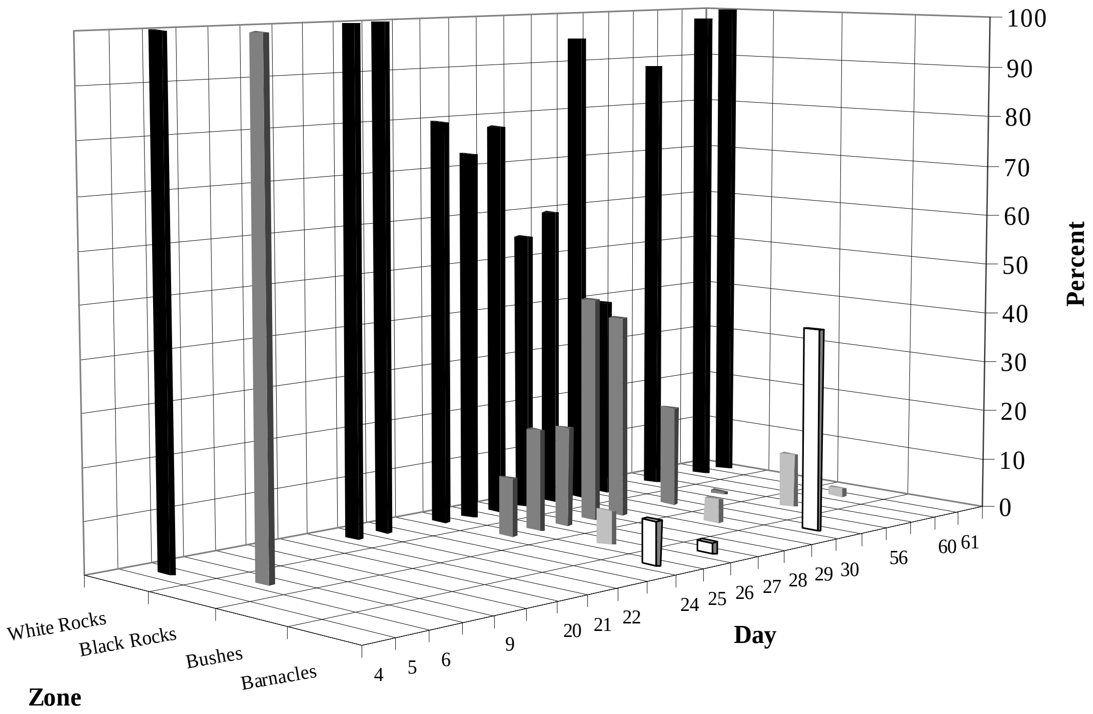
Relative Intertidal Location

Based on the information provided in the following six figures (Figure 31 - Figure 36), no clear persistent trends or changes in observed intertidal location are seen. There may be a recurring pattern of observations of more or fewer individuals located lower in the intertidal zone on different days, but those changes may be attributed to relatively consistent observation periods and the tides affecting when the iguanas could venture out to eat (since hatchlings and females only eat during low tides). Greater detail on zones identified during observations is located in Figure 2 (on page 16) and the accompanying text prior to the figure.



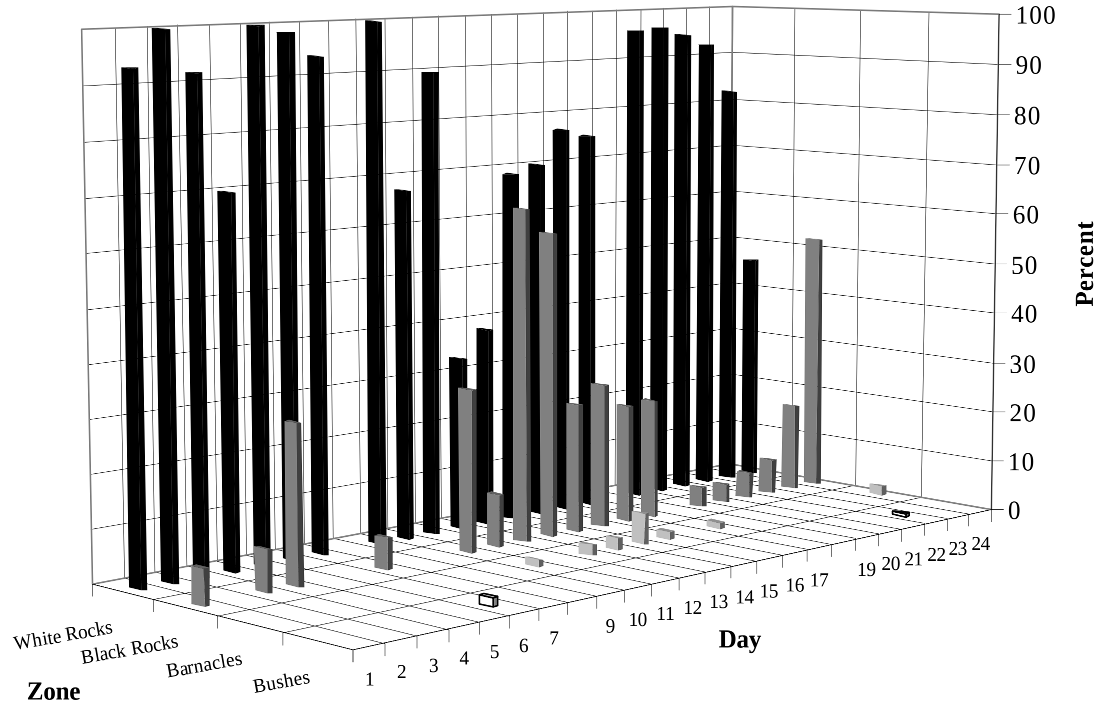
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
☐ Barnacles	13	7	2	6	1	5	5	1		27		1	4	2	1		1	2	3	1	1	0		0	1	2	5	10		13				
░ Bushes	3	6	6	7	5	5	1	4	2	3	6	5	4	7	6		7	12	7	8	10	11		10	7	7	6	13	34	13		13		
▓ Black Rocks	48	26	28	20	28	37	41	34	6	18	12	19	24	21	19		16	27	17	15	14	14		19	24	28	18	23	3	22		6		
■ White Rocks	38	61	65	67	66	53	53	61	92	51	82	74	69	70	74		76	60	73	76	75	74		71	68	64	71	54	63	52		81		100

Figure 31. Relative intertidal location of territorial males from daily observations during the 1997 reproductive season. See text for detailed descriptions of zones. Numbers in table are rounded off.



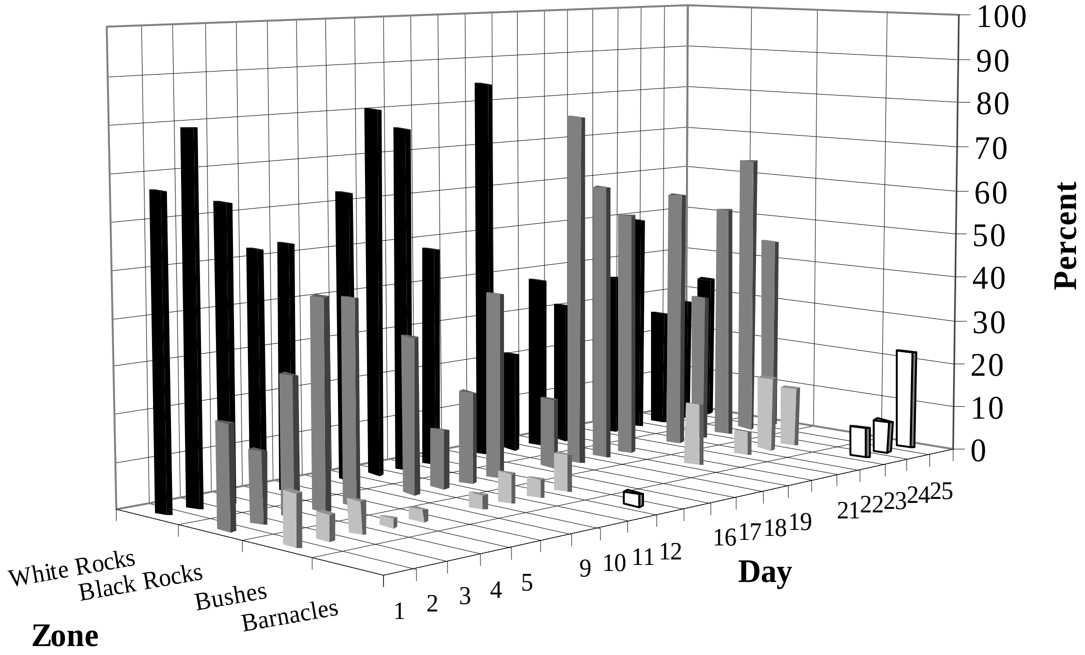
	4	5	6		9		20	21	22		24	25	26	27	28	29	30		56		60	61
□ Barnacles											9		2				40					
░ Bushes												7				5			11		2	
▒ Black Rocks			100								11	20	20	44	40		20		1			
■ White Rocks		100						100	100		80	73	78	56	60	95	40		89		98	100

Figure 32. Relative intertidal location of females from daily observations during the 1997 reproductive season. See text for detailed descriptions of zones. Numbers in table are rounded off.



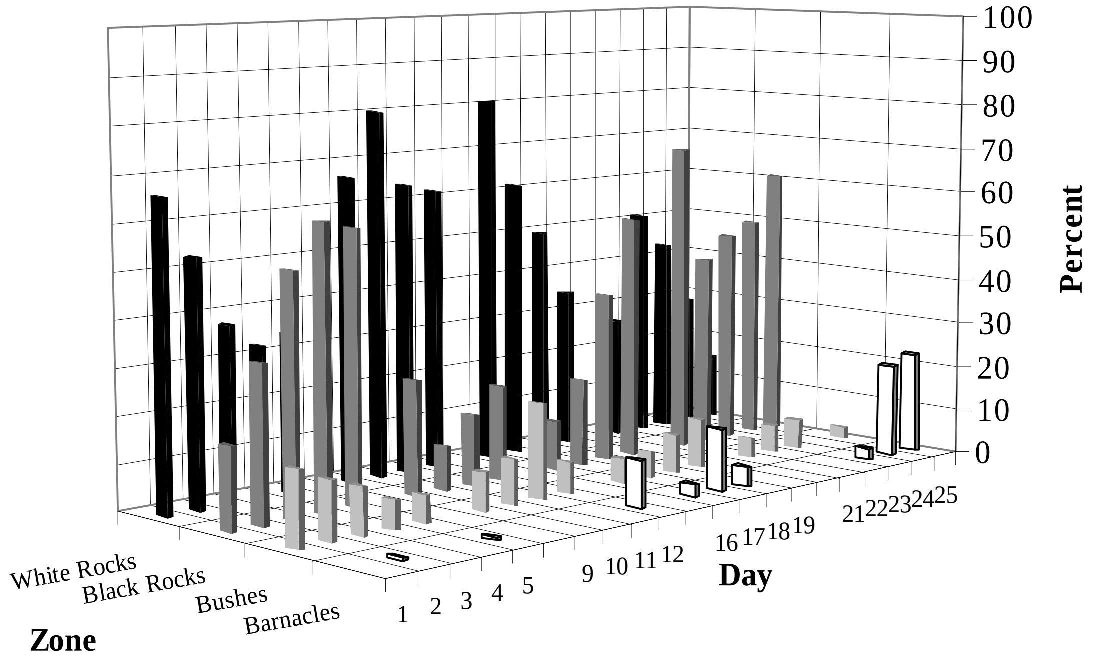
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
☐ Bushes						2																1		
▒ Barnacles										1		2	2	6	2		1							2
▓ Black Rocks	7		8	30			6			31	10	64	59	25	28	23	23		4	4	5	7	17	52
■ White Rocks	93	100	92	70	100	98	94		100	68	90	34	39	69	70	77	75		96	96	95	93	83	46

Figure 33. Relative intertidal location of hatchlings from daily observations immediately following hatching in 1997. See text for detailed descriptions of zones. Numbers in table are rounded off.



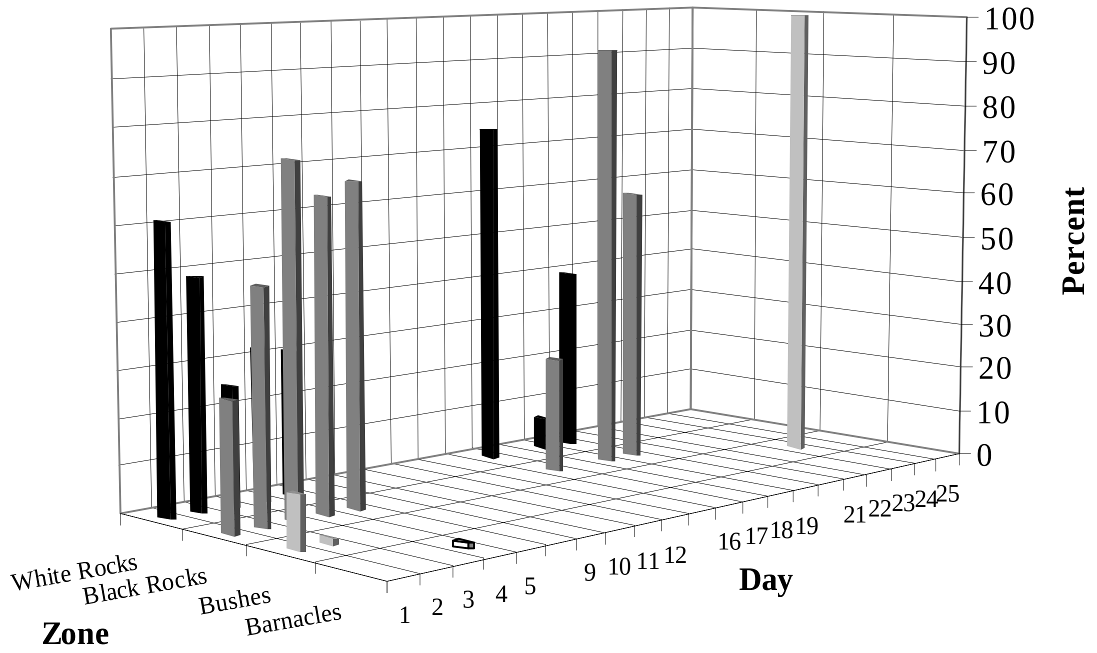
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
☐ Barnacles										3									7	7	22
░ Bushes	11	5	7	2	3		3	6	4	8					14		5	17	13		
▒ Black Rocks	22	15	30	45	44		34	13	20	41		15	78	62	55		58	33	53	64	44
■ White Rocks	67	79	63	53	53		63	81	76	49		85	22	38	32		37	50	27	29	33

Figure 34. Relative intertidal location of territorial males from daily observations during the 1998 reproductive season. See text for detailed descriptions of zones. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
☐ Barnacles		1			1					10		3	13	4					3	20	22
░ Bushes	16	13	10	6	6		8	10	21	7		5	6	8	11		4	6	6		2
▒ Black Rocks	18	34	52	61	59		25	10	16	21		11	19	38	54		69	43	47	50	60
■ White Rocks	66	53	38	33	35		67	80	64	62		81	62	50	35		27	51	44	30	15

Figure 35. Relative intertidal location of females from daily observations during the 1998 reproductive season. See text for detailed descriptions of zones. Numbers in table are rounded off.



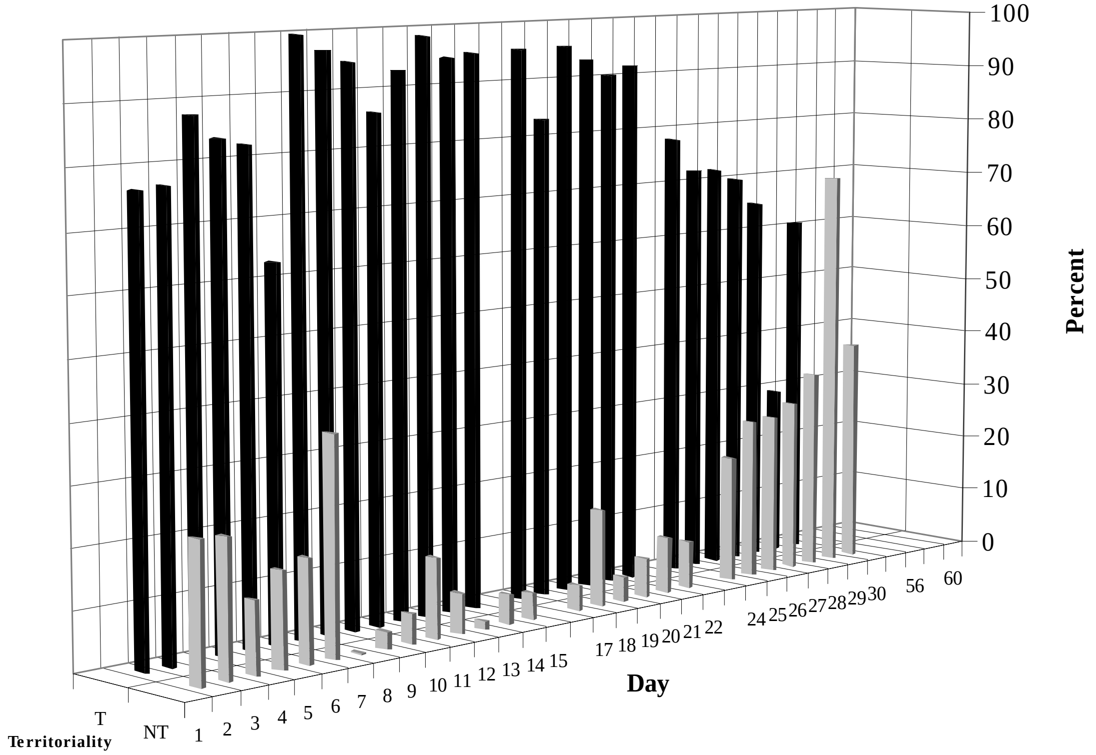
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
☐ Barnacles				1																	
░ Bushes	11	1																	100		
▒ Black Rocks	27	49	74	66	69							25		93	60						
■ White Rocks	61	49	26	33	31							75		7	40						

Figure 36. Relative intertidal location of hatchlings from daily observations during the 1998 reproductive season. See text for detailed descriptions of zones. Numbers in table are rounded off.

Male Territoriality Observations

During the 1997 reproductive season, males marked as territorial were classified during each observation as either exhibiting or not exhibiting territorial behavior. Examples of behavior classified as not territorial would be eating, swimming, walking around low in the intertidal zone, or laying prone on a rock during generally active times of the day. Territorial behaviors included headbobbing, fighting with neighboring males, copulating, attempted copulation, or pre-copulatory activities such as side-walking, or sitting attentively on a prominent rock.

As with intertidal location (see page 65), Figure 37 appears to indicate there may be a cyclical pattern affecting the numbers of males exhibiting territoriality. This may be due to some foraging needs or something else not recognized. However, near the end of observations there was a steady increase in the amount of behavior classified as not territorial. This was occurring at the same time that many marked males were observed wandering around and leaving the islet (see Figure 9 on page 39).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
▨ NT		23	23	12	16	17	37	0	3	5	14	7	1	5	5		4	17	4	7	10	8		22	28	28	30	35	70	39				
■ T		77	77	88	84	83	63	100	97	95	86	93	99	95	95		96	83	96	93	90	92		78	72	72	70	65	30	61				

Figure 37. Daily percentage of marked (previously as territorial) males observers identified as displaying territorial behavior during the 1997 reproductive season. Territoriality is identified as follows (see text for complete descriptions): T-territorial, NT-not territorial. Numbers in table are rounded off.

Behavior Frequencies

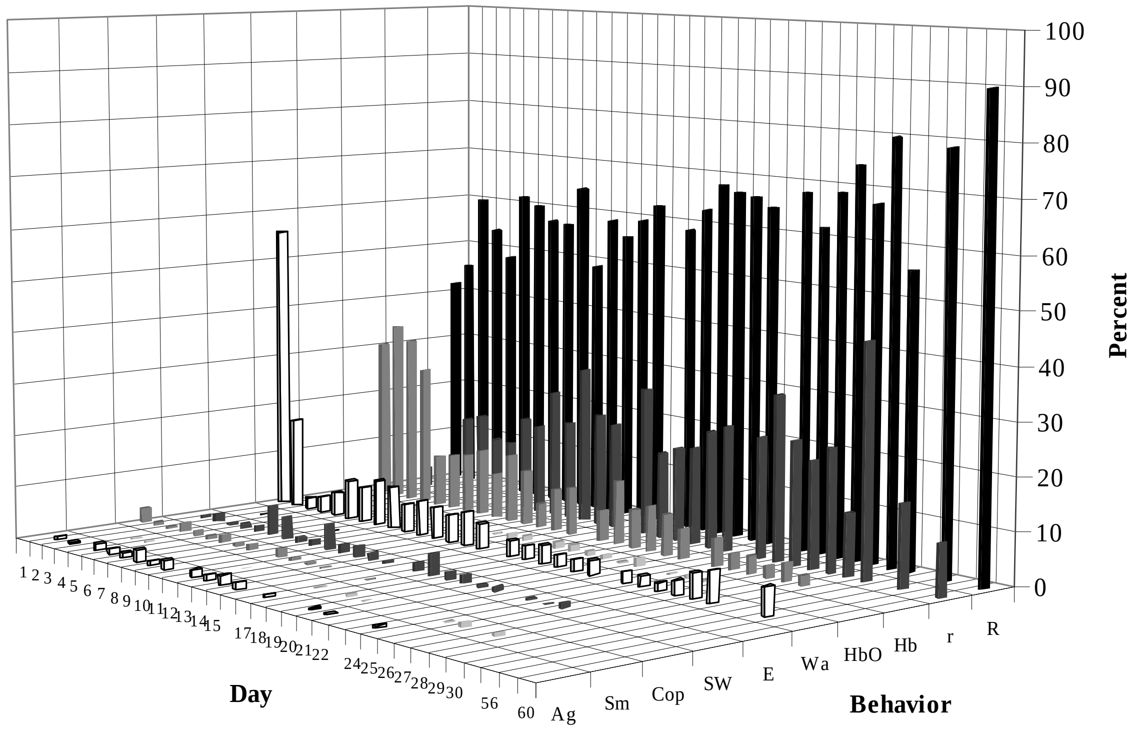
Changes in observed behavior with time are presented in the following six figures (Figure 38 - Figure 43). Table 1 (page 17) lists the behaviors recorded along with abbreviations used. When examining these figures, it is important to have the total unique individuals plots in mind as well (Figure 9 - Figure 14, beginning on page 39).

Males marked as territorial during 1997 (Figure 38) engaged in less territorial (Hb, HbO, and Ag) and reproductive (SW and Cop) activity as time passed, while frequency of rest position observations increased with time. Marked females during the 1997 reproductive season (Figure 39) displayed no reproductive or aggressive behavior. However, it is important to note that females marked during the period were marked only after they had been observed copulating. As noted elsewhere, this would indicate that copulation occurs only once per reproductive season. Female headbobbing after copulation is considered a signal of having already copulated. Only large males were observed headbobbing with an open mouth.

Immediately after hatching in 1997, hatchlings (Figure 40) appeared to have some cyclical pattern of rest behavior. These apparent cycles may have something to do with tides and available feeding and digestion periods.

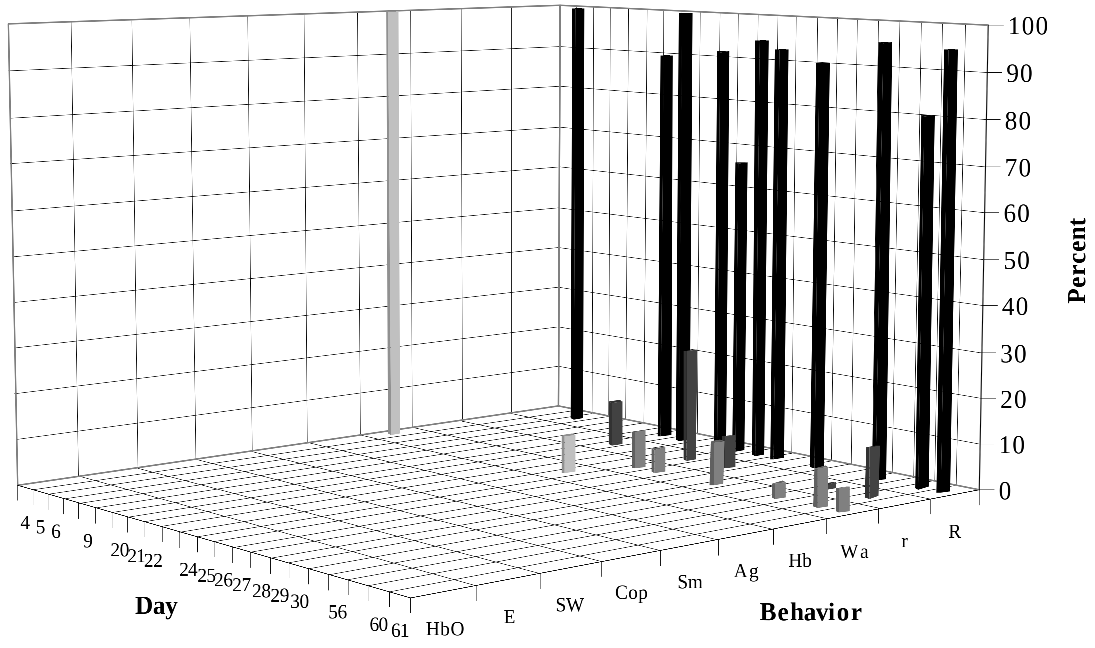
Contrasting the observed behaviors of males marked as territorial during the reproductive seasons of 1997 (Figure 38) and 1998 (Figure 41) provides some indication of how numbers (or density) and physical condition of individuals affect behavior. During 1998, little agonistic, aggressive, or territorial behavior (Ag, Hb, and HbO) was observed. At the same time, almost no reproductive behavior (SW and Cop) was recorded. Physical condition of both males and females had deteriorated between the two seasons. (Physical comparisons of groups between years appear later, with figures beginning on page 109.) Additionally, any territorial or reproductive behaviors observed in 1998 were recorded at the very beginning of observations and tapered off quickly.

During 1998, none of the previously marked females were observed copulating or headbobbing (which would have indicated copulation had occurred). Few hatchlings from the previous year were observed in 1998. Observations of those that did remain only identified various forms of rest and walking.



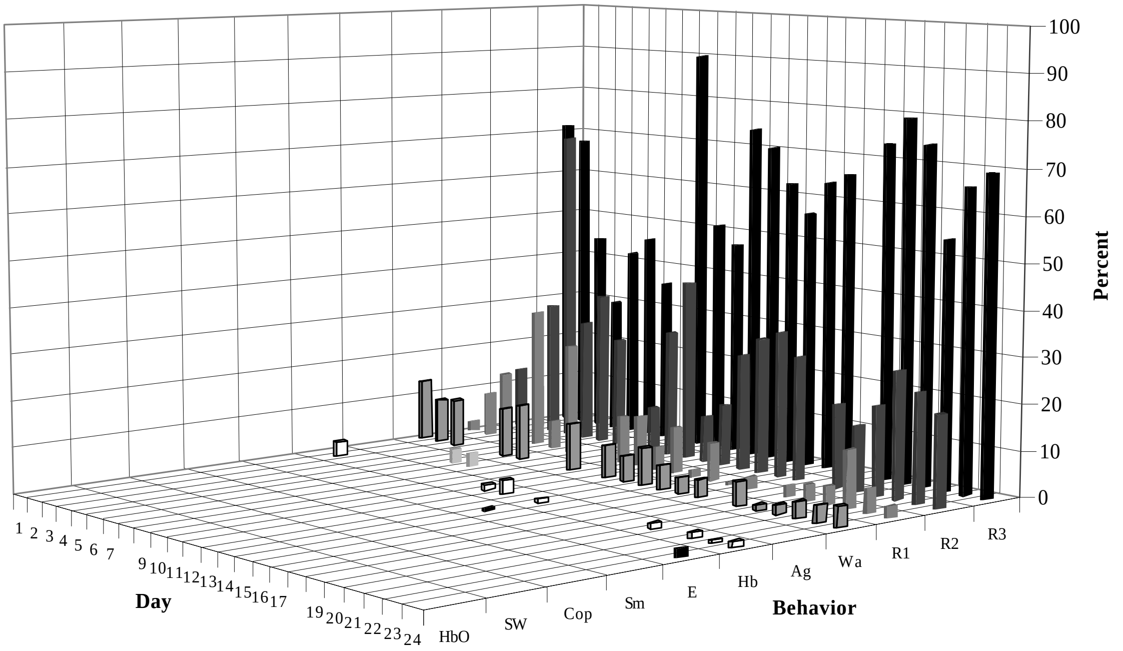
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
□ Ag		1	0		1	1	1	2	1	2		1	1	2	1		0			0	0			0										
░ Sm				0	0							0					0		1	0					0	1		1						
▒ Cop	3	1	0	2	1	1	2	1	1		2	1	0	0			0																	
▓ SW		1	2	0	1	1	6	4	1	1	5	2	2	1	1		2	4	1	2	1	1		0	0	1								
■ E			0					0																										
□ Wa	56	17	2	3	4	8	7	9	8	5	7	6	5	6	5		3	3	4	2	2	3		2	2	2	3	5	6			5		
░ HbO						1	1	2	1	1		2	0	1	1		1	1	1	1	0	2		0	1	0								
▒ Hb		31	35	33	27	10	11	11	13	9	13	11	5	8	9		6	12	7	9	8	6		5	3	4	2	4	2					
▓ r		4	0	8	16	17	13	13	18	17	25	19	30	22	20		28	16	18	19	22	24		23	32	24	21	23	12	44		16		10
■ R	42	46	60	54	49	62	60	57	57	65	49	59	56	60	63		59	63	68	67	67	65		68	62	69	74	67	80	56		79		90

Figure 38. Territorial male behavior patterns as a proportion of all observed daily behaviors during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



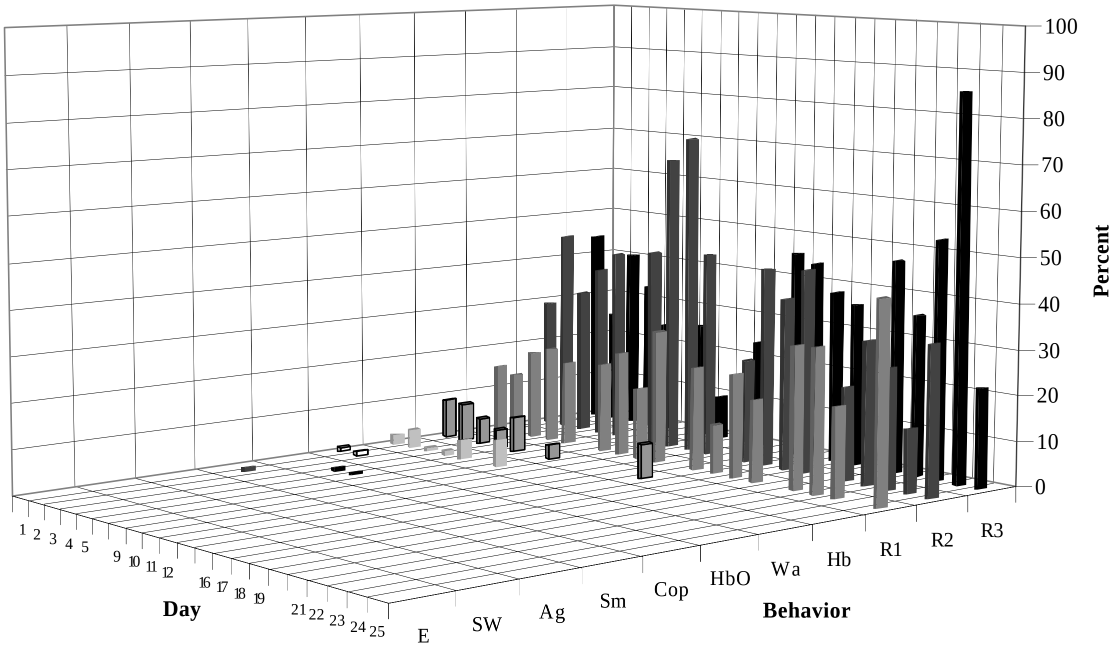
	4	5	6		9		20	21	22		24	25	26	27	28	29	30		56		60	61
☐ HbO																						
■ E																						
■ SW																						
■ Cop																						
■ Sm																						
☐ Ag																						
■ Hb	100										8											
■ Wa												8	5			10			3		8	5
■ r								10				25		7					1		11	
■ R			100					90	100		92	67	95	93		90			96		81	95

Figure 39. Female behavior patterns as a proportion of all observed daily behaviors during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



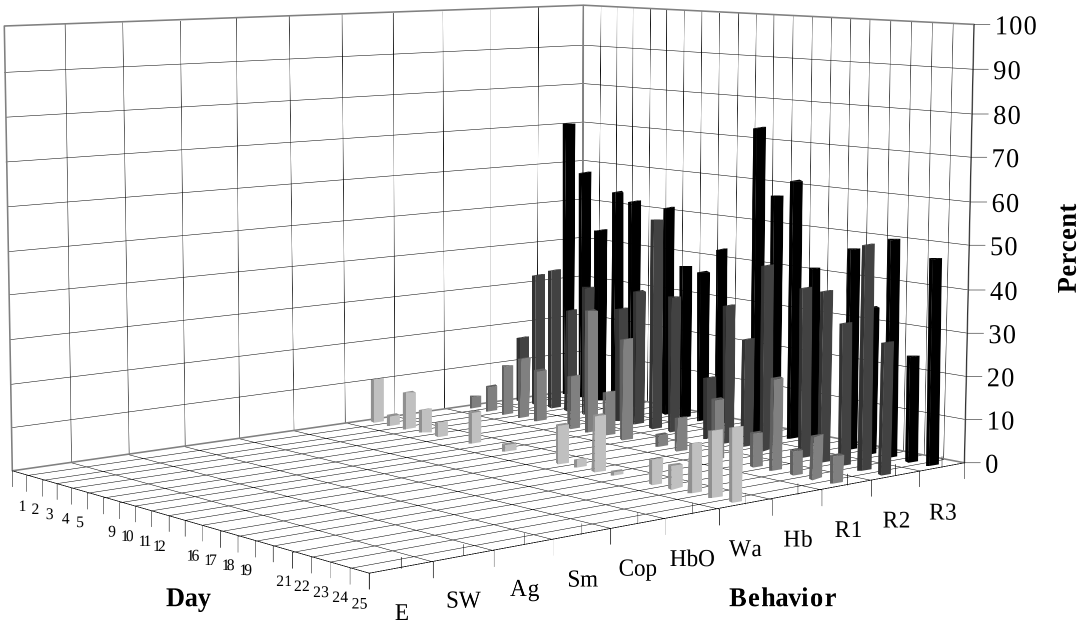
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
□ HbO																								
■ SW																								
■ Cop																								
■ Sm																								
■ E														1										2
□ Hb		3									1	3		1						1		1	1	1
■ Ag						3	3																	
■ Wa	13	10	10			11	12			10		7	6	8	5	3	4		5	1	2	4	4	5
■ R1	2	10	15		31	6	24			10	11	5	10	2	8	1	3		3	4	4	13	5	3
■ R2	13	10	30	70	27	34	24		10	28	40	10	14	26	30	32	27		18	14	20	28	24	20
■ R3	71	68	45	30	42	46	36		90	51	48	74	70	63	57	64	66		74	80	74	55	66	69

Figure 40. Hatchling behavior patterns as a proportion of all observed daily behaviors immediately following hatching in 1997. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



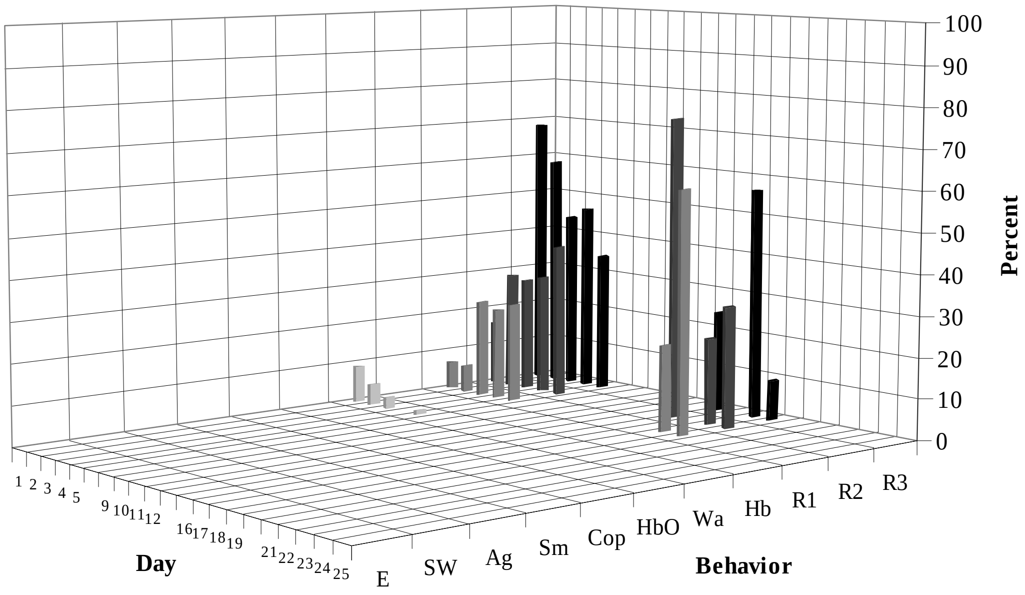
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
░ SW																					
▒ Ag																					
▓ Sm		1																			
■ Cop				1	1																
□ HbO	1	1																			
░ Wa	2	4	1	1	4		6														
▣ Hb	9	9	6	4	8		3					8									
▒ R1	15	14	20	21	19		20	23	16	30		23	11	23	18		32	32	20		44
▓ R2	29	46	33	39	43		45	67	72	46		23	44	38	45		21	32	27	14	33
■ R3	44	26	41	34	25		26	10	12	24		46	44	38	36		47	36	53	86	22

Figure 41. Territorial male behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
■ SW																					
■ Ag																					
■ Sm																					
■ Cop																					
□ HbO																					
■ Wa	11	3	9	6	3		8		2			9	2	13	1		5	5	11	15	16
■ Hb																					
■ R1	3	6	12	15	13		13	30	11	24		3	8		14		8	21	5	10	6
■ R2	16	33	35	25	32		28	33	51	33		15	33	26	44		40	39	33	51	30
■ R3	70	58	44	54	52		52	38	37	43		74	58	62	42		47	34	51	24	48

Figure 42. Female behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
■ SW																					
■ Ag																					
■ Sm																					
■ Cop																					
□ HbO																					
■ Wa	9	5	3		1																
■ Hb																					
■ R1	7	7	24	23	25									21	60						
■ R2	16	29	29	30	39							75		21	30						
■ R3	68	59	44	47	35							25		57	10						

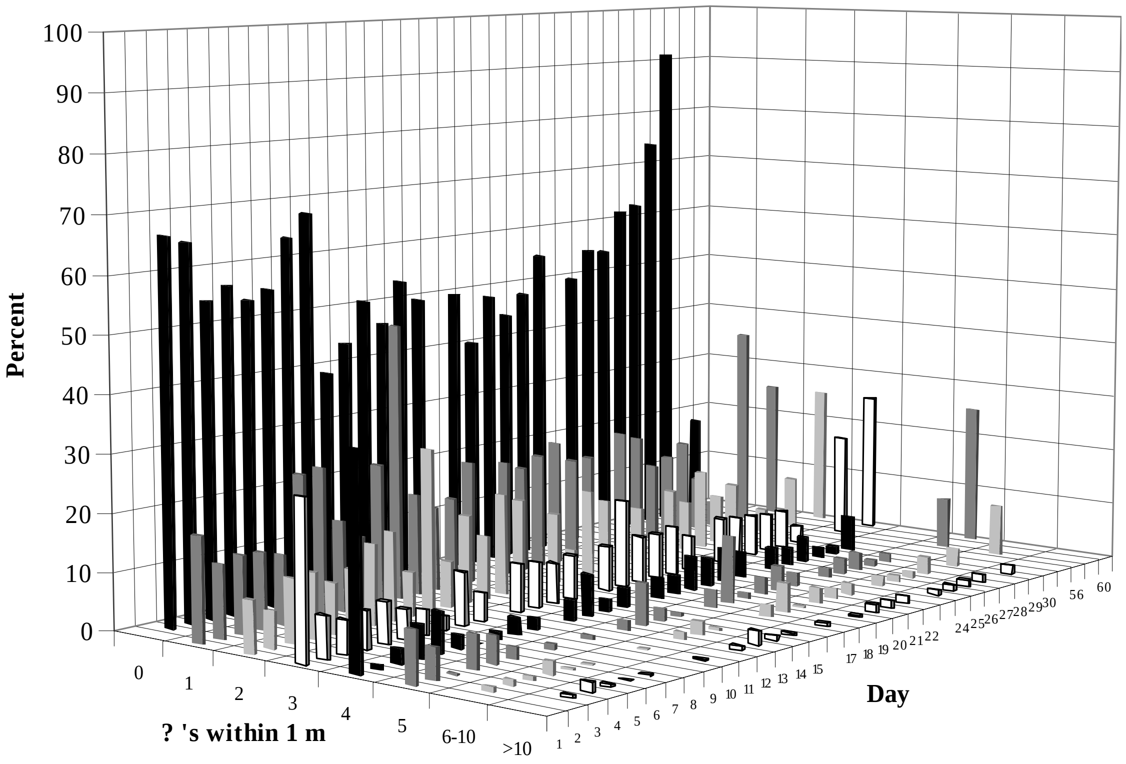
Figure 43. Hatchling behavior patterns as a proportion of all observed daily behaviors during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.

Grouping Tendency

In the following six figures (Figure 44 - Figure 49), percentage of individuals with a given number of either females or all iguanas within 1 m is plotted daily. The first two figures (Figure 44 and Figure 45) record number of female sized iguanas within 1 m, while the other figures record the total number of iguanas within 1 m. However, since most iguanas around males marked as territorial during 1997 were female sized (actual sex unknown, but of compatible size) the information is considered comparable, enabling a general conclusion to be drawn.

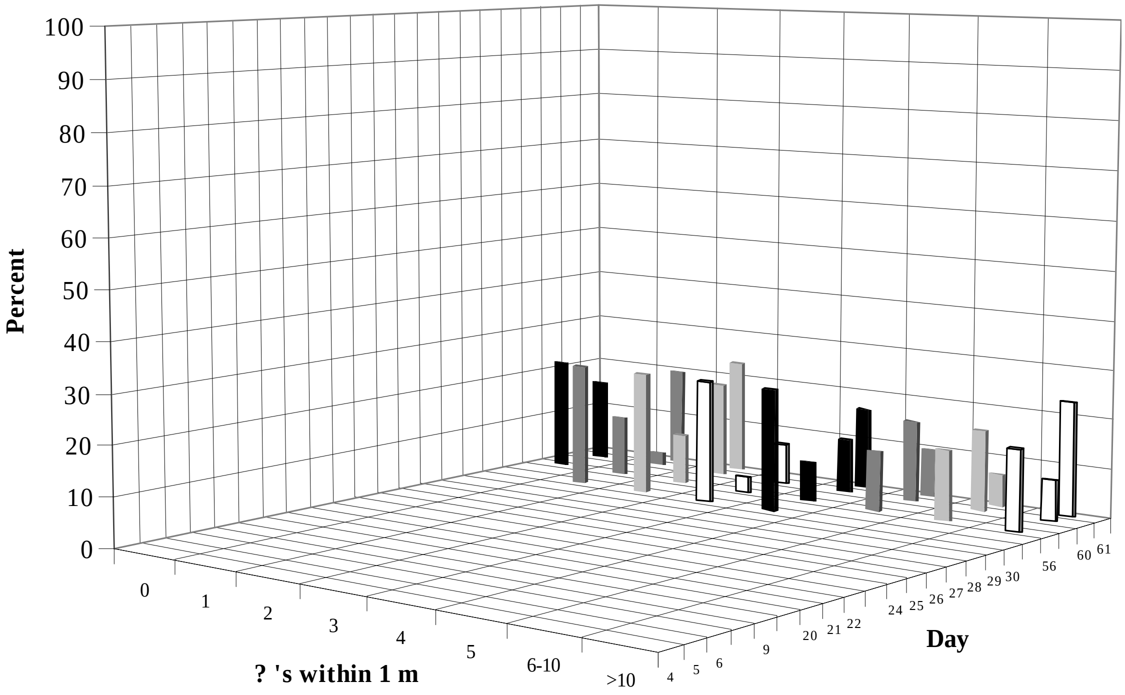
When all six figures are compared it seems quite clear that males marked as territorial during the 1997 reproductive season had fewer other iguanas nearby (within 1 m) than any other category of iguanas at any other observed time. Other groups have fairly even distributions of marked individuals found within each category. Behavior of marked territorial males in 1997 is believed to have contributed most to the change in frequency of categorical numbers of individuals nearby (see Figure 38 and Figure 41, beginning on page 76), since percentages of categorical numbers of nearby iguanas for males marked as territorial in 1998 is comparable to females and hatchlings during the same period and behavior of males was generally less territorial and aggressive in 1998.

Hatchling grouping tendencies may also have changed (or been selected for) between the time they hatched and were initially observed and marked and when they were later observed during the 1998 reproductive season. In 1997 (Figure 46) more solitary hatchlings were observed compared to 1998 (Figure 49). Infrequent and sporadic observation later in the season is given as a reason for the much different frequencies seen late in the observation period.



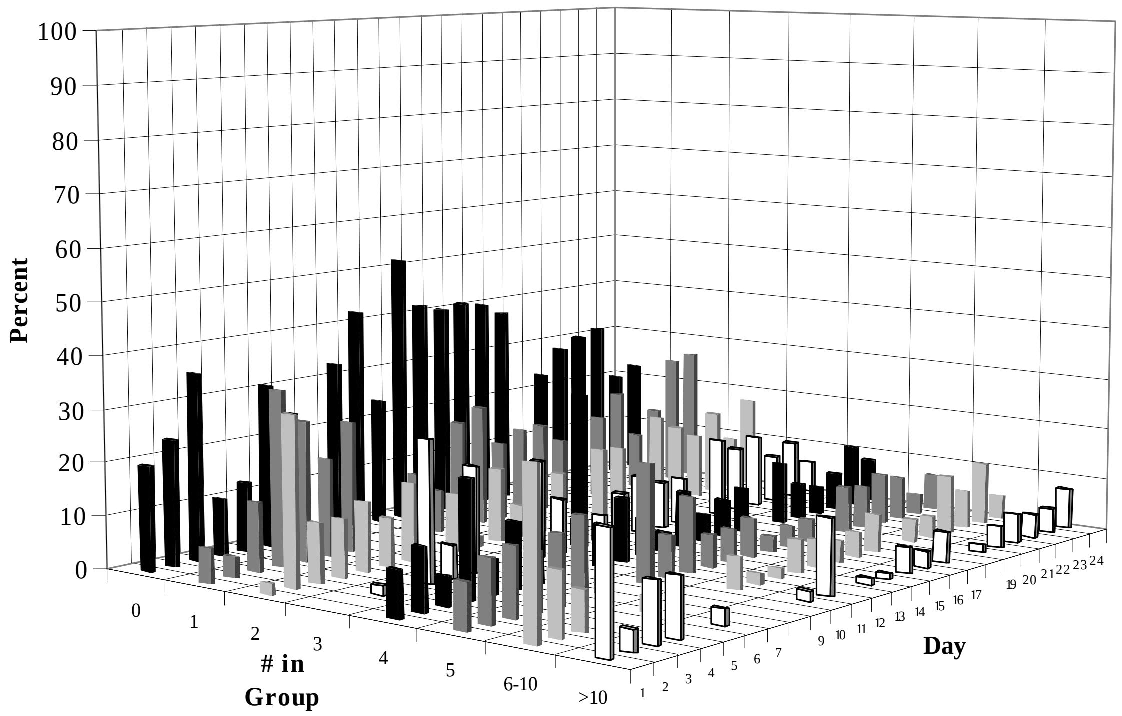
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
□ >10			0	2	0	0	0			0		1	2	1	0		1		0	1	1	1		1	1	1	1		2					
▨ 6-10		1	1	1	2	0	0			0		1	2	1			2	5	0	3	2	2		2	1	1	3		3			9		
▩ 5	9	5	0	6	5	2		1		1		2	7	2	1		3	12	1	3	4	2		2	3	3	1	1				9		25
■ 4	36	1	3	6	7	3	0	1	3	2		4	7	2	4		4	3	6	5	6	5		4	3	5	2	1	6					
□ 3	27	7	6	7	7	5	4	2	9	5		8	8	7	8		8	15	8	8	8	6		8	7	7	7	7	3			18		25
▨ 2	9	6	11	11	9	10	14	15	8	28	8	15	11	18	16		12	5	15	13	11	10		12	9	14	9	10	2	4		9		25
▩ 1	18	13	13	13	12	25	26	16	12	24	48	18	15	16	22		20	19	20	22	18	18		22	20	14	15	17	10	4		36		25
■ 0		66	65	55	57	54	55	64	68	39	44	51	47	54	50		50	41	49	45	48	55		50	55	54	62	63	74	91		18		

Figure 44. Time based pattern indicates percent of all territorial males observed during a single day that had a categorical number of females within 1 m during the 1997 reproductive season. Numbers in table are rounded off.



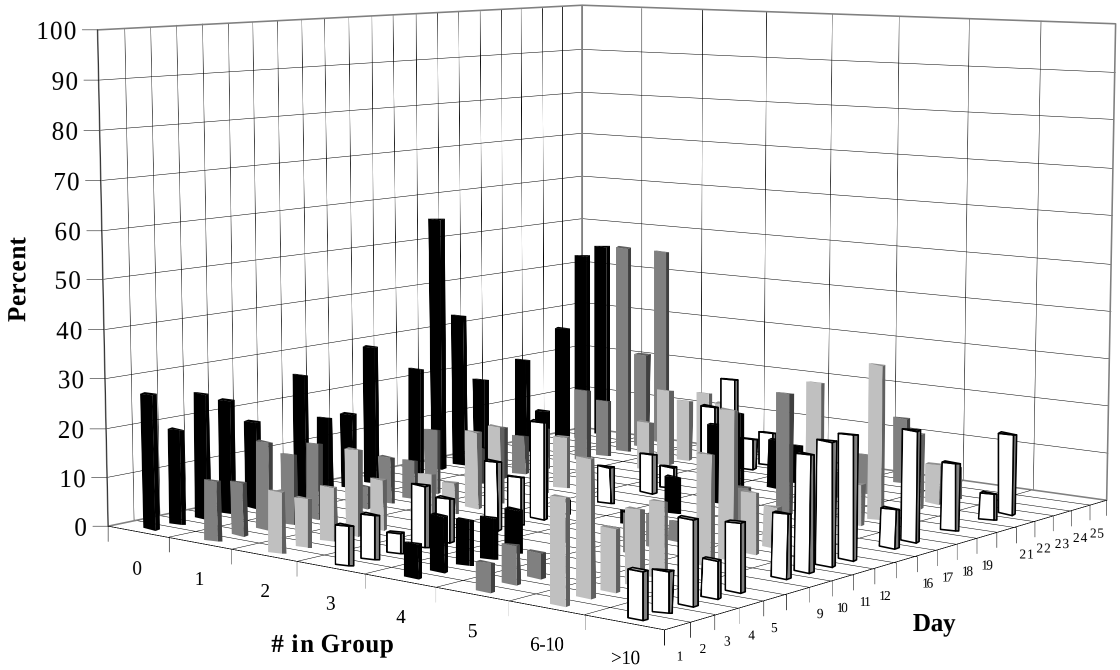
	4	5	6		9		20	21	22		24	25	26	27	28	29	30		56		60	61
□ >10																			16		8	23
░ 6-10																			14		17	7
▒ 5																			12		17	10
■ 4																	25		8		11	17
□ 3																	25		3		8	
░ 2																	25		10		19	23
▒ 1																	25		12		3	20
■ 0																			23		17	

Figure 45. Time based pattern indicates percent of all females observed during a single day that had a categorical number of females within 1 m during the 1997 reproductive season. Numbers in table are rounded off.



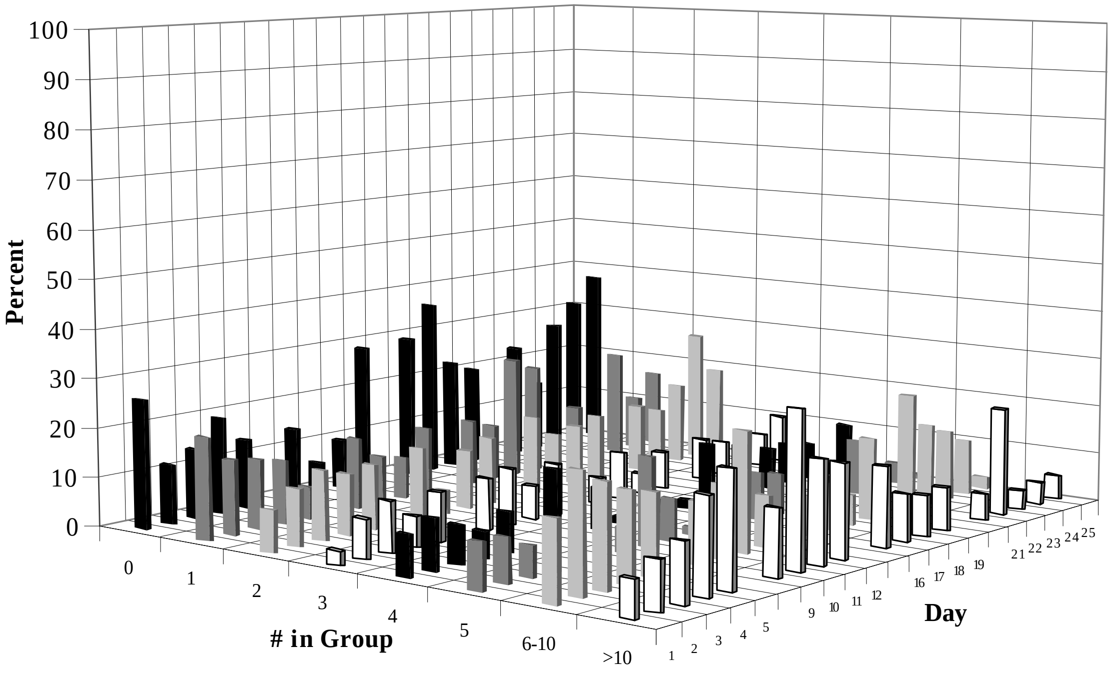
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
□ >10	22	4	11	11		3				2	14		1	1	5	3	6		1	4	6	5	5	8
▒ 6-10	31	12	8			3				6	2	2	6	5	4	5	7		4	4	11	7	12	5
▓ 5	9	12	13	11	13	16	5		22	8	14	6	6	8	3	4	4		9	8	10	8	4	7
■ 4	9	12	6	22	7	13	10		33	8	12	7	4	11	5	7	9		12	7	6	7	12	8
□ 3			2		27	6	20			18	10	5	5	9	11	9	9		15	12	14	9	11	6
▒ 2	2	32	11	11	13	9	15		11	2	14	6	12	11	13	14	13		18	15	13	16	10	18
▓ 1	7	4	13	33	27	19	25			12	8	21	23	15	17	17	13		16	21	11	15	25	26
■ 0	20	24	36	11	13	31	25		33	43	24	52	42	41	41	41	38		24	29	31	32	21	22

Figure 46. Time based pattern indicates percent of all hatchlings observed during a single day that had a categorical number of other iguanas within 1 m immediately following hatching in 1997. Numbers in table are rounded off.



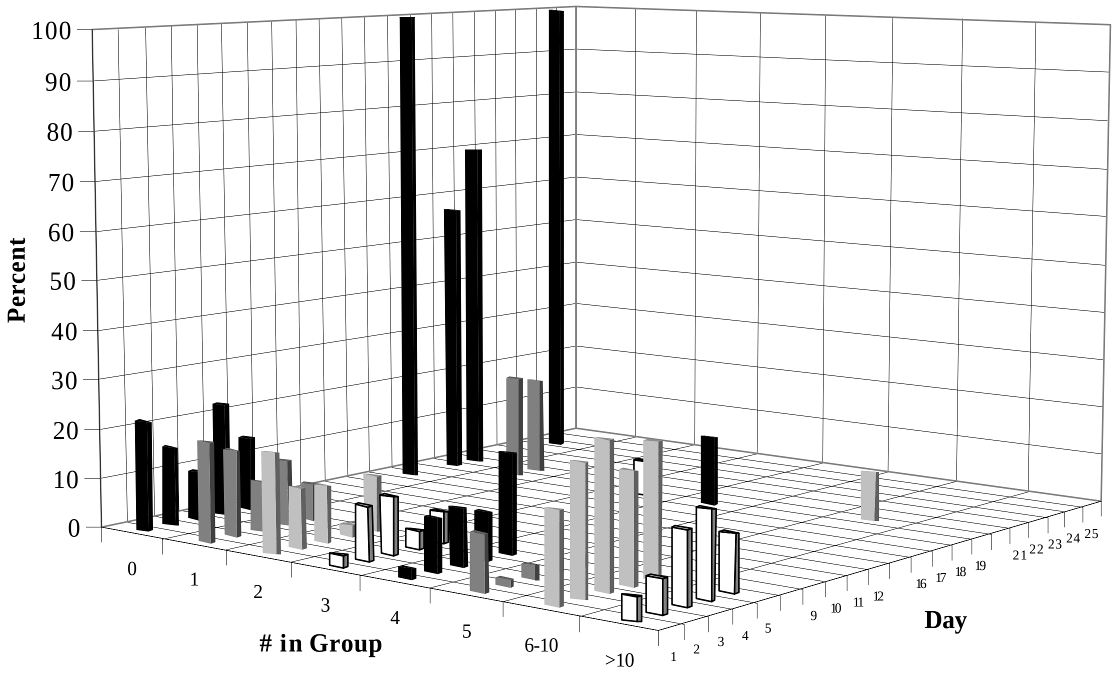
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ >10	9	8	16	7	13		12	23	24	24		8	22		14		5	17			
░ 6-10	20	26	12	14	15		22	29	12	8		31		8	32		16	8	7		
▒ 5	5	8	5	3	11		8	6	4	3		8		25	5		5	8		14	
■ 4	7	11	9	8	9		5			3		8		17	18		11	8			
□ 3	8	9	4	12	9		14	10	20	3		8		8	5		16	21	7	7	
░ 2	12	10	11	17	10		9	6	16	16		8	11				11	17	13	14	11
▒ 1	12	11	18	14	16		5	10	8	14		8	11	8	9		16	13	47	21	44
■ 0	27	19	26	23	18		26	16	16	30		23	56	33	18		21	8	27	43	44

Figure 47. Time based pattern indicates percent of all males observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ >10	8	10	12	19	23		14	31	21	19		16	10	8	9		5	22	4	5	5
▒ 6-10	16	24	21	18	16		13	15	24	10		8	10	6	17		24	17	14	11	2
▓ 5	9	9	6	6	8		5	18	9	2		8	10	8	7		4	11	3	5	1
■ 4	8	10	8	6	8		15	3	9	2		3	2	13	3		9	9	8	2	10
□ 3	3	8	10	6	10		10	11	7	10		5	10	4	8		9	7	5	7	10
▒ 2	8	12	14	12	13		15	5	12	14		16	12	13	14		14	12	17	27	19
▓ 1	21	15	14	13	7		15	10	9	14		14	12	25	23		12	8	22	11	16
■ 0	26	12	14	20	14		15	7	10	29		30	37	23	21		24	15	27	32	37

Figure 48. Time based pattern indicates percent of all females observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ >10	4	7	14	17	11																
░ 6-10	18	25	29	22	26										10						
▓ 5	11	1	3	10	3																
■ 4	2	11	11	10	20									14							
□ 3	2	11	11	4	6									7							
░ 2	20	12	11	2	11																
▓ 1	20	17	10	13	8									21	20						
■ 0	22	16	10	23	15							100		57	70				100		

Figure 49. Time based pattern indicates percent of all hatchlings observed during a single day that had a categorical number of other iguanas within 1 m during the 1998 reproductive season. Numbers in table are rounded off.

Behaviors Associated with Grouping

Association of behavior with grouping is examined in the next twelve figures (Figure 50 - Figure 61). Table 1 (page 17) lists the behaviors recorded along with abbreviations used. Figures are paired. First a figure displaying the daily mean number of either females or all iguanas within 1 m that is associated with each recorded behavior is given. It is followed by a figure showing the daily proportion of all observed grouped individuals that were found within 1 m of iguanas displaying each of the recorded behaviors.

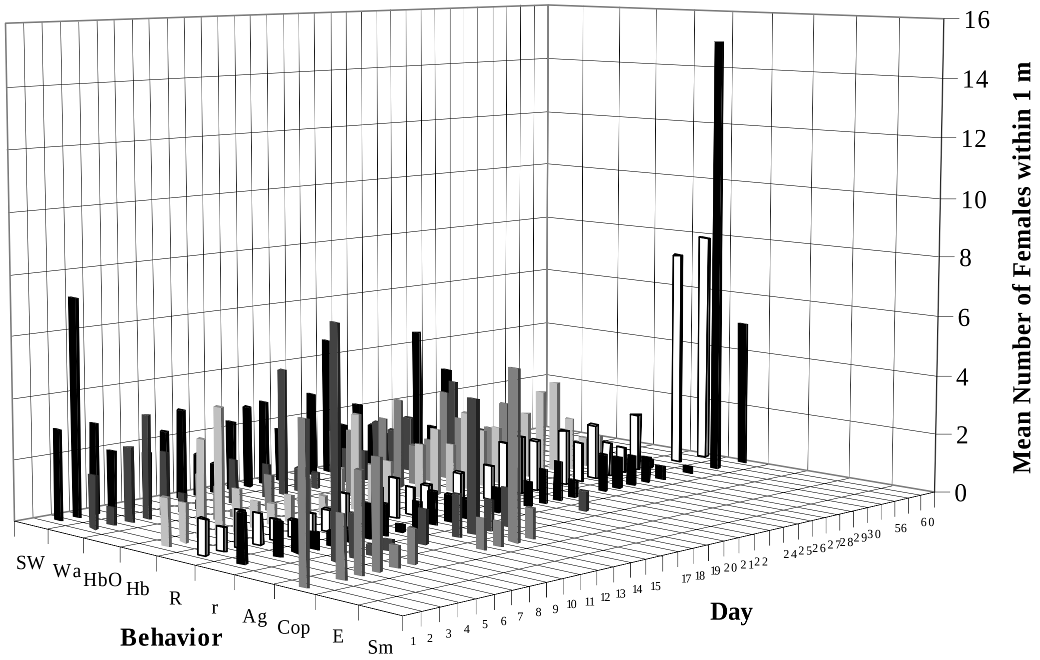
As in the previous section (Grouping Tendency) information was recorded in two ways. For marked males and females during the 1997 reproductive season number of female sized iguanas within 1 m was recorded while observations after that recorded the total number of all iguanas within 1 m. However, since most iguanas within 1 m were always female sized (exact sex unknown but of compatible size) the plotted final results are considered comparable.

Males marked as territorial during the 1997 reproductive season had a relatively even (across behaviors) number of females within 1 m (see Figure 50). However, there were some notable “spikes” in concentration of female sized iguanas associated with reproductive or territorial behavior (SW, Ag, and Cop). The overall mean numbers of females within 1 m of males during 1997 was generally much lower than the recorded number of all iguanas within 1 m in 1998. This is particularly interesting considering the lower overall numbers of iguanas on the islet in 1998, attributed to ENSO conditions, and the difference in recorded male behavior frequencies between the reproductive seasons of 1997 and 1998 (see Figure 38 on page 76 and Figure 41 on page 79). Explanations could range from the few males observed in 1998 having access to more densely grouped iguanas to iguanas not being driven away as much due to less aggressive or reproductive activity exhibited by males in 1998. A few points that support the latter explanation are the increase in number of female sized iguanas within 1 m of males marked as territorial following the most active period of the reproductive season in 1997. On days 56 and 60 (Figure 50) only rest behavior was observed and there were much higher mean numbers of female sized iguanas at that time. Marked males present at that time have been considered “full time” residents of the islet, unlike others that swim over from Isla Santa Cruz during the reproductive season. Figure 51 indicates that as time passed a higher proportion of female sized iguanas near marked males was associated with rest behavior. This is likely due to an increase in resting behavior among marked males (see Figure 38 on page 76). An equivalent, but downward, trend associated with changes in marked male behavior is found when looking at the proportion of female sized iguanas found near headbobbing (Hb) males and comparing that with change in behavior over time found on Figure 38 (page 76). The 100% of female sized iguanas associated with male copulatory behavior on the first day of observation is attributed to too little recorded information concerning group numbers at that time.

The few observations of marked females during the 1997 reproductive season do not provide enough information to detect any changing trends, but comparing the mean numbers of female sized iguanas grouped around particular behaviors during the same period as that recorded for marked territorial males provides somewhat of a baseline to make overall comparisons of the two groups by. In general there appear to be 3-4 times as many female sized iguanas near resting females (Figure 52) as near males exhibiting any type of behavior (Figure 50). The large difference in proportion of females observed near one type of resting female over another (Figure 53) is attributable to the higher frequency of R behavior during the whole period (see Figure 39 on page 77).

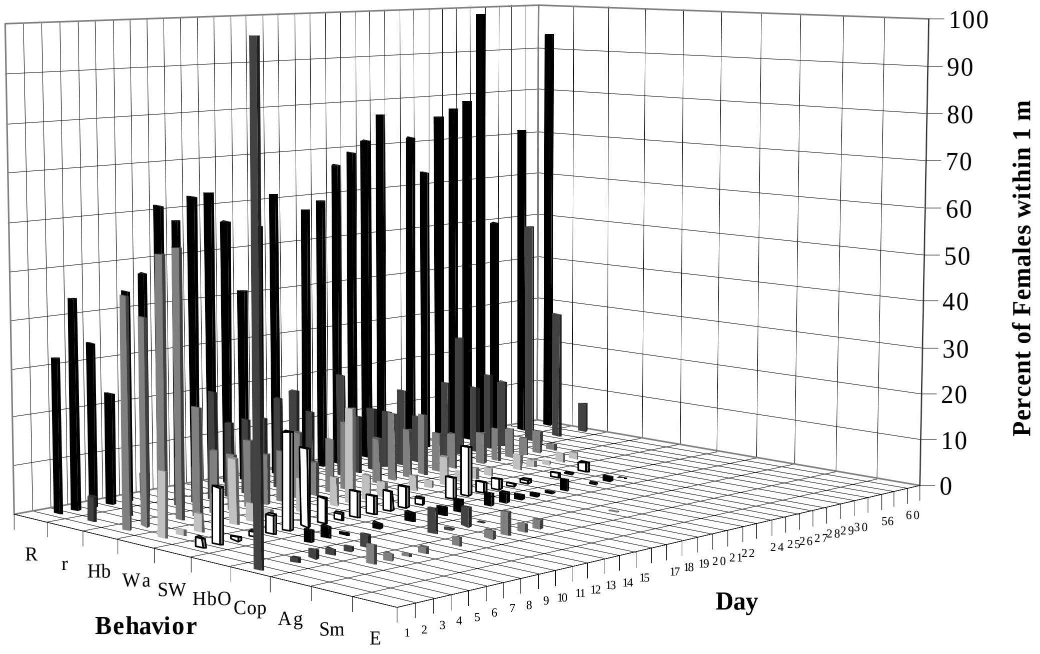
Immediately following hatching in 1997 hatchlings were observed resting in all three positions among groups of iguanas of approximately equal mean size (Figure 54), however, overall a higher proportion of grouped iguanas was found near hatchlings resting in position R3 (Figure 55), which reflects the fact that R3 was the most commonly observed behavior (see Figure 40 on page 78) at the time. Headbobbing (Hb), apparently used by adult males and females for territorial or warning communication, was observed relatively frequently, but the most striking observation was of agonistic behavior (Ag) occurring in groups where mean number of other iguanas within 1 m was relatively high (9-15). In 1998 no Hb behavior was observed in the few individuals seen and there seemed to be a shift during the period of observation in the frequency of resting type exhibited in aggregations from the more alert R3 to the more prone R1 (Figure 61). This is also reflected in the chart of all hatchling behavior (see Figure 43 on page 81).

A comparison of mean number of individuals within 1 m of territorial males and marked females exhibiting grouped behaviors during the 1997 and 1998 reproductive seasons is provided in Figure 62 (page 105). In 1997 number of “female sized” iguanas was recorded while in 1998 total number of all iguanas was recorded. However, extremely few iguanas (1-3%) present in 1998 were not considered “female sized.” All rest behaviors were classified as type 0. Behaviors associated with copulation (Cop and SW) were classified as type 2. Remaining behaviors (Wa, Sm, Ag, Hb, HbO, E) were grouped as type 1. Statistically significant differences between mean number of individuals associated with behavior types were found between all in 1997 territorial males and between types observed in females during 1998. While differences in means for territorial males in 1998 are not significant an interesting trend is evident.



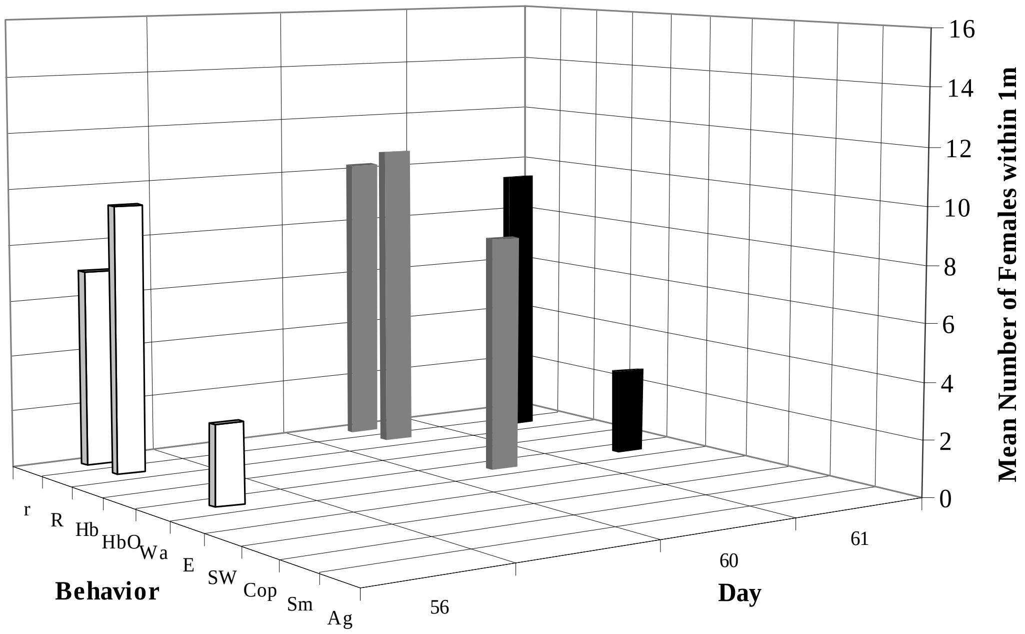
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
☐ Sm																																		
▨ E																																		
▨ Cop	5.0		2.0	3.2	3.5	0.7	1.1				1.0	0.8	5.5	1.0																				
■ Ag					7.3	1.4	0.3	0.4		1.1		1.4	4.3	1.0	1.3					0.7														
■ r		1.6		1.1	1.0	0.6	0.5	0.4	1.2	1.0	0.3	0.9	1.1	0.9	0.7		0.9	1.4	0.9	1.2	1.3	0.6		1.3	1.1	1.0	0.9	0.5		0.3		15.		5.0
☐ R		1.1	0.8	1.2	1.0	0.7	0.6	0.7	0.7	1.1	0.6	1.1	1.3	0.9	0.9		1.1	1.8	1.1	1.8	1.9	1.7		1.8	1.3	1.8	1.1	0.9	2.0	0.2		7.4		7.9
▨ Hb		1.6	1.3	3.2	4.1	1.4	0.9	0.7	0.9	1.3	0.7	1.5	3.2	1.4	1.4		1.8	2.2	1.6	2.6	1.9	1.9		2.2	2.9	3.1	1.8	1.0	1.0					
▨ HbO						1.6	1.1	0.3		1.2		1.2		2.3	2.2		2.5	3.0	1.3	1.4	3.0	2.0		1.5	2.3	0.5								
■ Wa		1.8	0.6	2.5	3.4	2.1	0.7	0.3		1.5		1.1	4.2	0.8	0.5		1.1	1.0	1.9	1.6	1.9	0.8		2.9	0.8	1.0	0.8	0.3						
■ SW		3.0	7.2	3.0	2.0	1.9	1.7	2.4	3.0	1.4	1.0	2.4	2.8	2.8	1.8		2.9	4.7	1.6	2.2	1.4	1.0		4.5	1.0	3.0								

Figure 50. Territorial male behavior patterns related to mean number of females within 1 m in daily observations during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



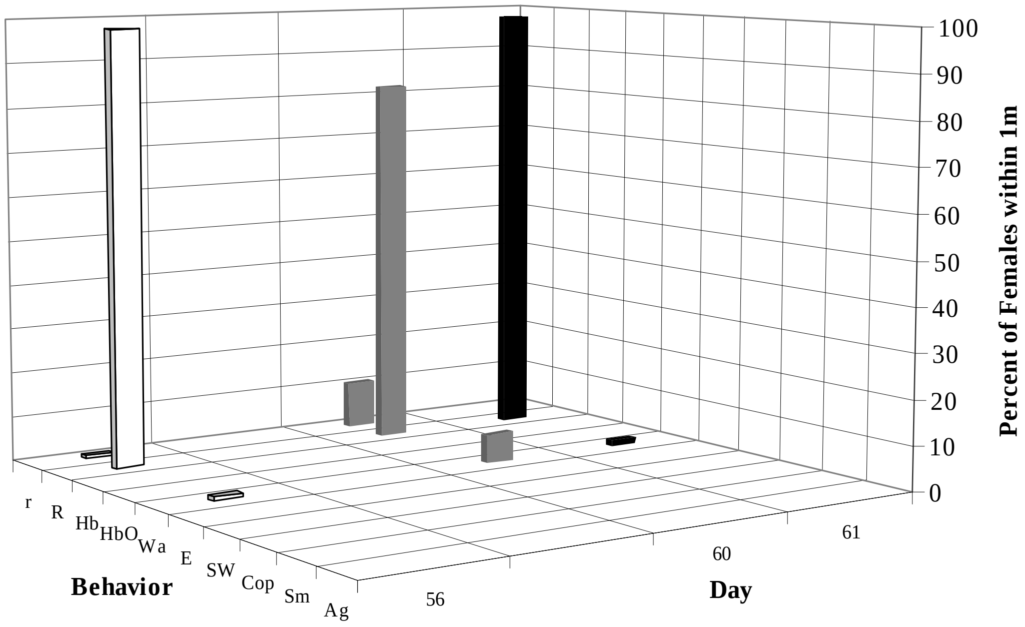
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		17	18	19	20	21	22		24	25	26	27	28	29	30		56		60
□ E																																		
░ Sm																																		
▒ Ag					4	1	1	1		2		2	5	2	2					0														
▓ Cop	100		1	2	1	1	3				5	0	4	0																				
■ HbO						3	3	1		1		2		2	3		3	2	1	1	0	2		0	1	0								
□ SW		2	11	1	1	4	20	16	5	1	5	4	4	4	1		4	10	2	2	0	1		1	0	2								
░ Wa		14	1	4	7	14	4	2		7		6	20	5	3		3	1	6	2	2	2		4	1	1	2	2						
▒ Hb		47	42	55	56	23	13	12	14	11	11	14	7	11	14		10	15	10	13	8	8		7	7	7	4	5	1					
▓ r		5		5	8	11	10	7	23	16	16	15	19	20	15		22	12	14	12	17	10		17	27	15	17	15		50		29		7
■ R		32	44	34	23	44	48	61	58	63	63	57	41	55	62		58	59	67	69	72	77		72	63	76	77	79	99	50		71		93

Figure 51. Territorial male behavior patterns related to proportion of all females seen within 1 m in daily observations during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



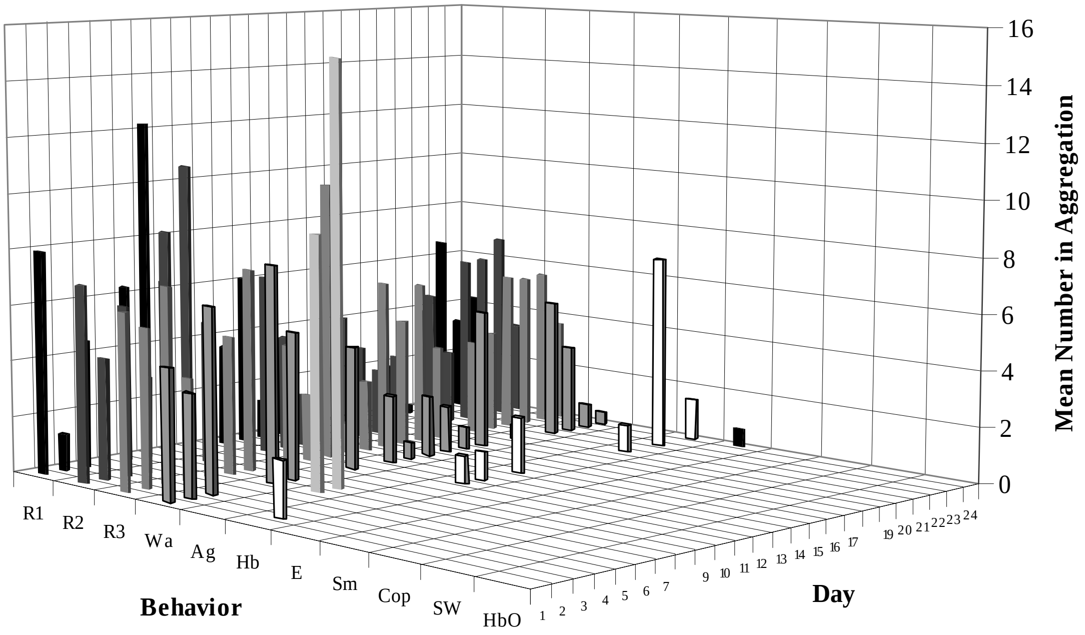
	r	R	Hb	HbO	Wa	E	SW	Cop	Sm	Ag
□ 56	7.0	9.6			2.8					
░										
▒ 60	10.2	10.9			8.3					
■ 61		9.6			3.0					

Figure 52. Female behavior patterns related to mean number of females within 1 m in daily observations during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



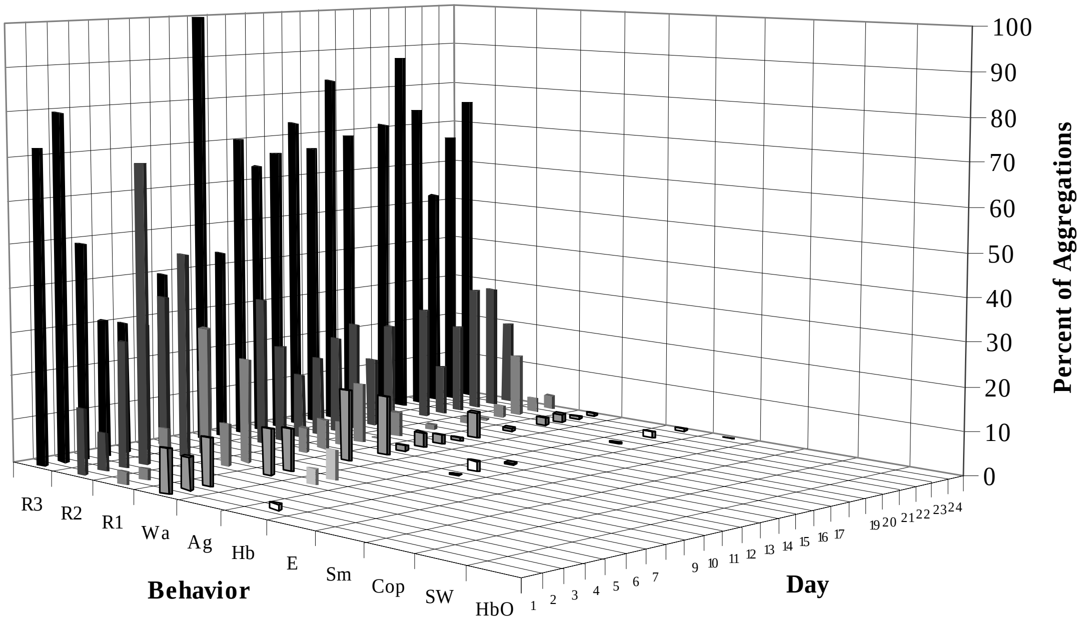
	r	R	Hb	HbO	Wa	E	SW	Cop	Sm	Ag
□ 56	1	98			1					
░										
▒ 60	11	83			7					
■ 61		98			2					

Figure 53. Female behavior patterns related to proportion of all females seen within 1 m in daily observations during the 1997 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R-resting, r-resting, Wa-walking. Numbers in table are rounded off.



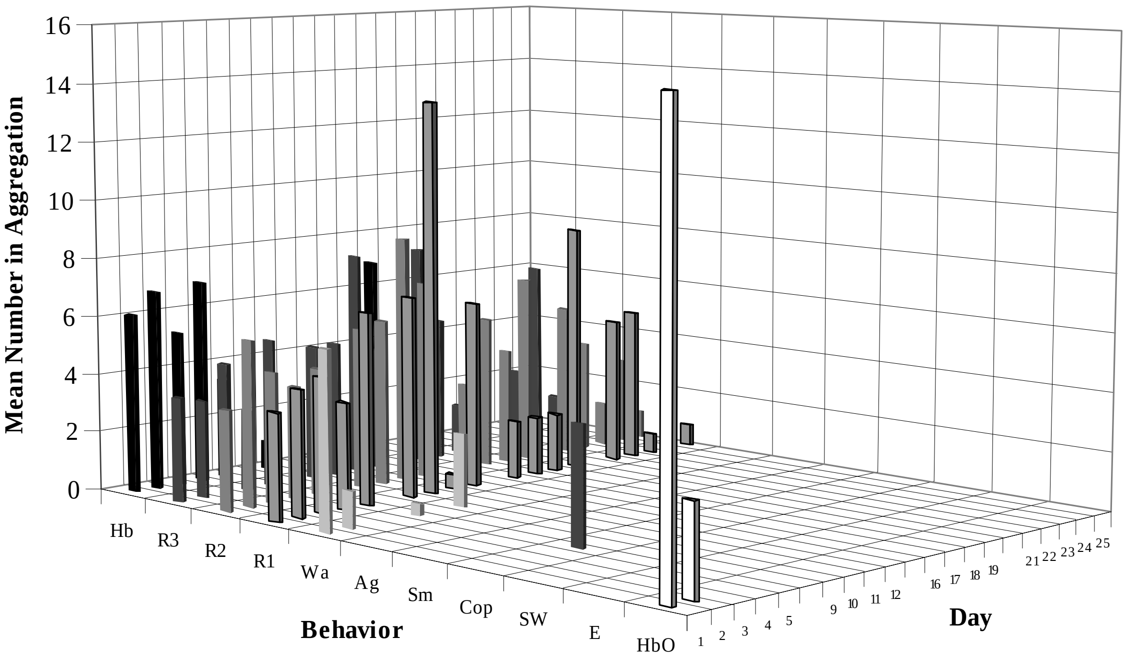
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
□ HbO																								
■ SW																								
■ Cop																								
■ Sm																								
■ E																								0.7
□ Hb		2.0									1.0	1.0		2.0						1.0		7.0		1.5
■ Ag						9.0	15.0																	
▣ Wa	4.7	3.7	6.6			7.7	5.3			4.4		2.4	0.6	2.2	1.7	0.8	5.0		0.5		5.0	3.2	0.9	0.4
■ R3	6.3	5.7	7.0	3.7	4.4	4.9	7.3		4.3	2.4	10.0	1.1	2.6	6.1	4.6	5.9	3.4		3.4	3.7	5.8	5.6	5.7	3.7
■ R2	7.0	4.3	6.1	3.4	8.6	10.9	5.1			6.5	4.2	1.9	3.7	4.6	3.4	2.4	2.8		5.0	2.7	6.2	6.2	6.9	3.4
■ R1	8.0	1.3	4.6		6.4	12.3	6.4			3.7	6.2	1.4	3.7	1.0	2.6		1.5		2.0	0.3	4.0	6.7	3.4	4.3

Figure 54. Hatchling behavior patterns related to mean number of iguanas within 1 m in daily observations immediately following hatching in 1997. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



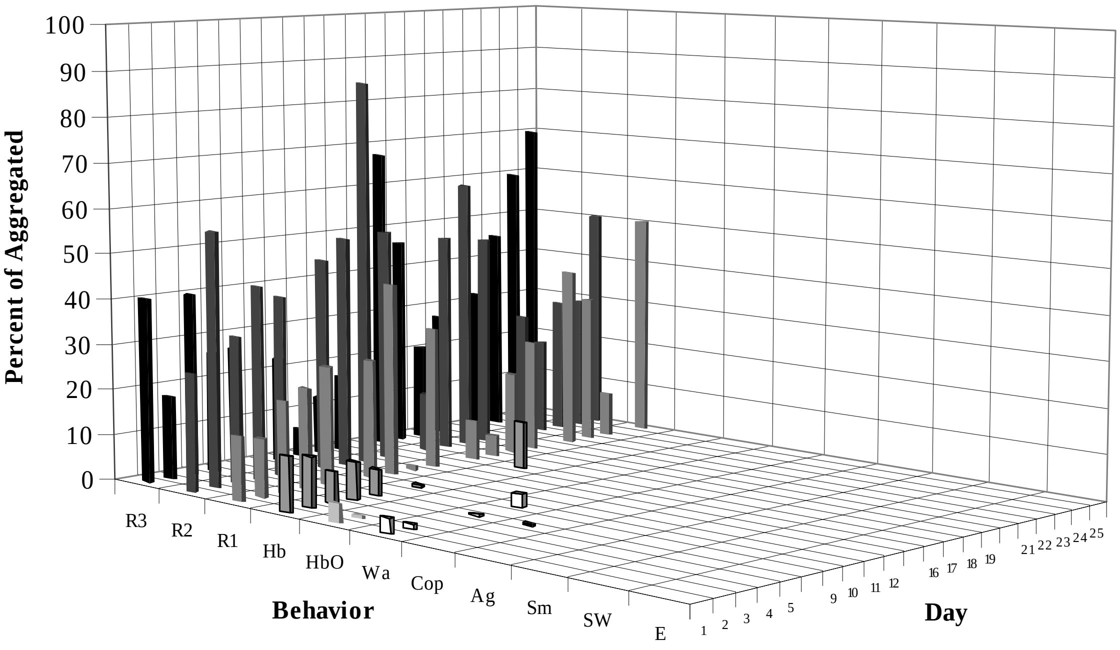
	1	2	3	4	5	6	7		9	10	11	12	13	14	15	16	17		19	20	21	22	23	24
□ HbO																								
■ SW																								
■ Cop																								
■ Sm																								
■ E																								0
□ Hb		1									0	2		0						0		1		1
■ Ag						4	7																	
▣ Wa	10	7	11			11	10			16		13	1	3	2	1	6		1		2	2	1	1
■ R1	3	3	11		32	10	24			6	7	6	14	0	6		1		2	0	3	15	3	3
■ R2	15	9	29	69	38	47	19			34	22	15	18	22	25	16	24		26	12	21	30	29	20
■ R3	72	80	50	31	30	29	40		100	44	71	64	67	73	67	83	69		71	88	74	52	67	75

Figure 55. Hatchling behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations immediately following hatching in 1997. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



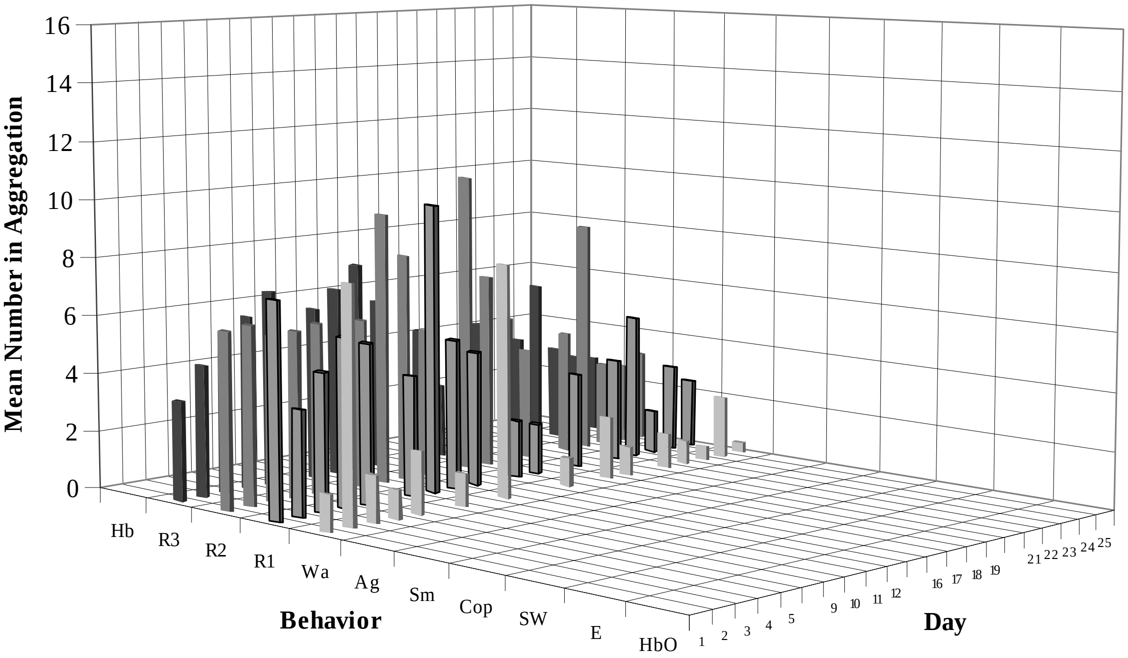
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ HbO	15.0	3.0																			
▨ E																					
▨ SW																					
■ Cop					4.0																
■ Sm																					
□ Ag																					
▨ Wa	6.0	1.3			0.4		2.5														
▨ R1	3.6	4.3	4.6	3.6	6.5		6.8	13.4	0.5	6.4		2.0	2.0	2.0	8.5		5.0	5.3	0.7		0.8
▨ R2	3.4	5.7	4.5	3.9	4.4		5.5	5.7	8.5	6.8		3.0	5.3	4.0	6.5		5.3	3.9	1.5	3.0	1.0
■ R3	3.6	3.3	4.5	2.8	5.1		4.6	4.7	7.7	4.2		7.7	5.0	1.8	4.8		2.7	6.4	1.5	1.2	
■ Hb	6.1	6.9	5.3	7.0	3.4		1.0					7.0									

Figure 56. Territorial male behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



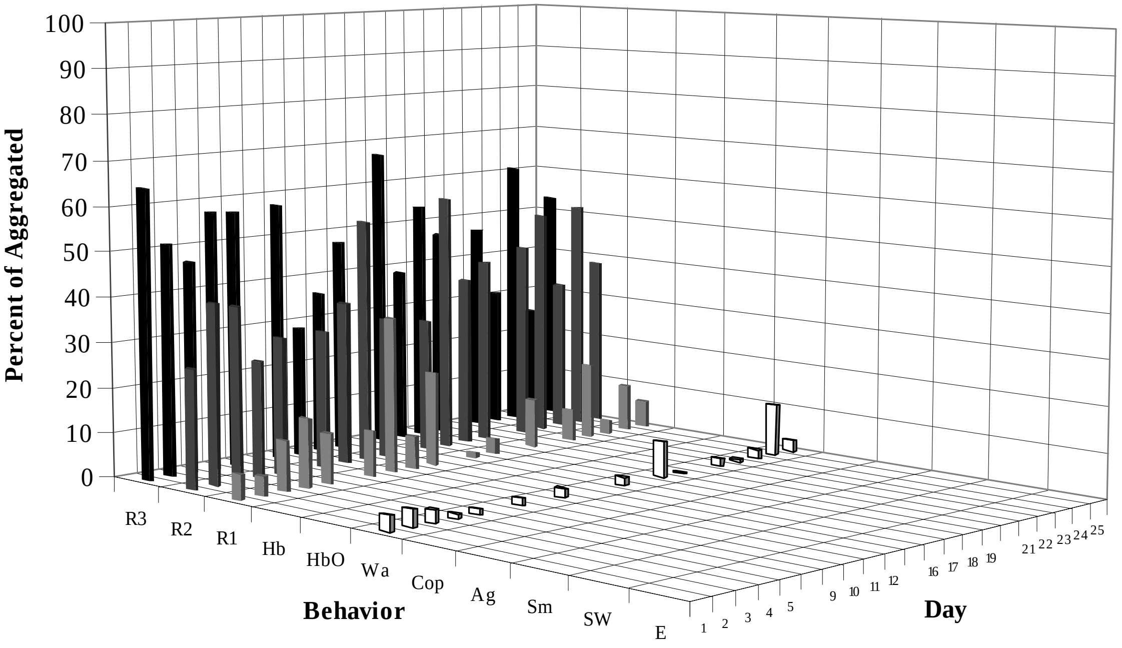
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
■ SW																					
■ Sm																					
■ Ag																					
■ Cop					1																
□ Wa	3	1			0		3														
■ HbO	4	1																			
▣ Hb	12	11	7	8	6		1					10									
■ R1	14	13	20	22	26		26	42	1	31		9	5	18	25		40	33	10		50
■ R2	26	56	32	43	40		47	51	86	52		13	49	61	47		28	21	30	30	50
■ R3	41	18	40	27	27		23	6	13	17		68	47	21	28		32	46	60	70	

Figure 57. Territorial male behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



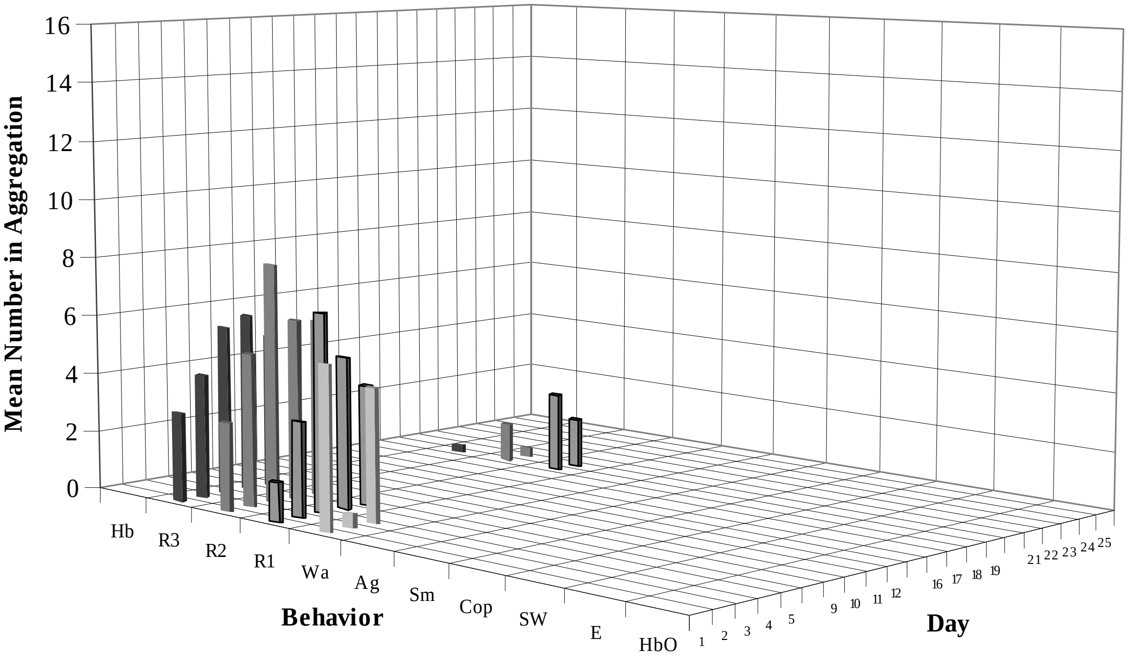
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ HbO																					
░ E																					
▒ SW																					
▓ Cop																					
■ Sm																					
□ Ag																					
░ Wa	1.3	8.0	1.6	1.0	2.2		1.1		8.0			1.0		2.2	1.0		1.2	0.8	0.5	2.2	0.4
▨ R1	7.3	3.6	4.7	5.8	5.5		4.2	9.9	5.2	4.6		2.0	1.8		3.3		3.6	5.0	1.5	3.0	2.4
▒ R2	6.1	6.2	5.7	5.8	5.9		5.8	9.4	7.9	5.2		10.4	6.8	5.2	3.9		4.3	8.2	3.0	2.8	3.1
▓ R3	3.4	4.5	5.5	6.0	6.8		6.0	6.6	7.3	6.0		4.7	2.6	3.0	4.6		3.8	5.7	3.3	2.9	2.7
■ Hb																					

Figure 58. Female behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



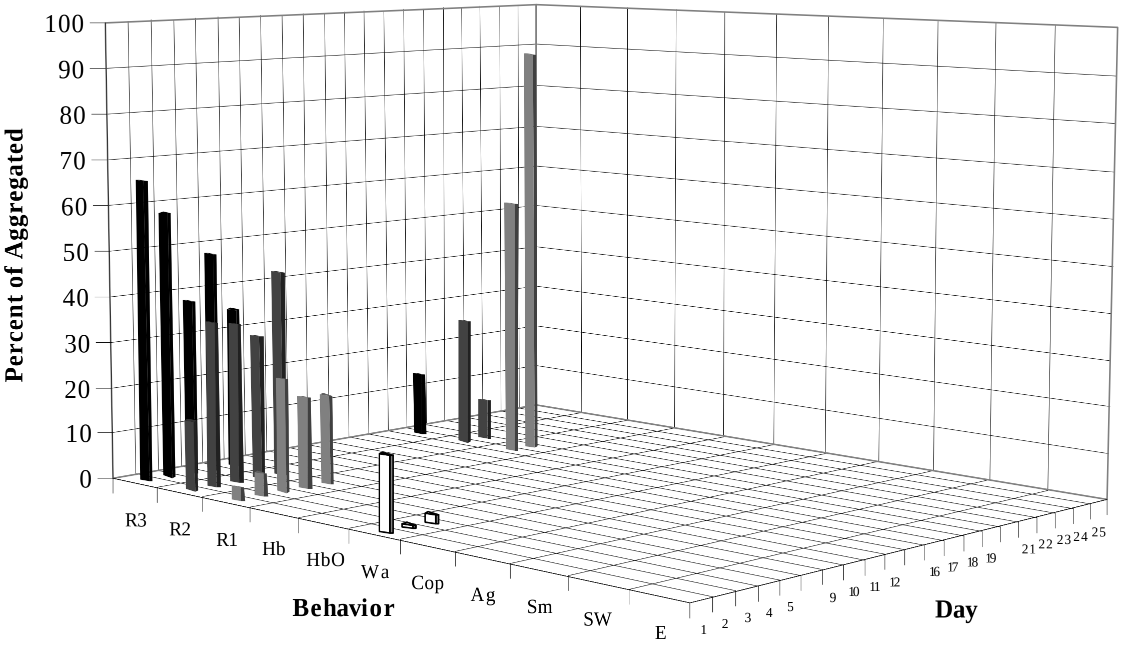
	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
■ SW																					
■ Sm																					
■ Ag																					
■ Cop																					
□ Wa	4	4	3	1	1		2		2			2		8	0		2	1	2	12	3
■ HbO																					
■ Hb																					
■ R1	6	4	11	15	11		10	34	7	21		1	4		11		7	17	3	11	6
■ R2	26	40	39	26	30		31	36	54	31		30	58	38	42		44	51	34	52	38
■ R3	64	52	47	58	57		58	29	36	48		67	39	54	47		47	31	61	26	53

Figure 59. Female behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ HbO																					
■ E																					
■ SW																					
■ Cop																					
■ Sm																					
□ Ag																					
■ Wa	5.5	0.5	4.5																		
▨ R1	1.3	3.2	6.7	5.1	4.1									2.7	1.7						
■ R2	3.0	5.2	8.1	6.1	6.0									1.3	0.3						
■ R3	3.0	4.2	5.7	6.0	5.2									0.3							
■ Hb																					

Figure 60. Hatchling behavior patterns related to mean number of iguanas within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.



	1	2	3	4	5		9	10	11	12		16	17	18	19		21	22	23	24	25
□ E																					
■ SW																					
■ Sm																					
■ Ag																					
■ Cop																					
□ Wa	16	1	2																		
■ HbO																					
■ Hb																					
■ R1	3	5	25	20	20									57	91						
■ R2	15	36	35	32	45									29	9						
■ R3	66	58	38	48	35									14							

Figure 61. Hatchling behavior patterns related to proportion of all aggregated iguanas seen within 1 m in daily observations during the 1998 reproductive season. Behaviors are abbreviated as follows (see text for complete descriptions): Ag-agonistic, Cop-copulating, E-eating, Hb-headbobbing, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking. Numbers in table are rounded off.

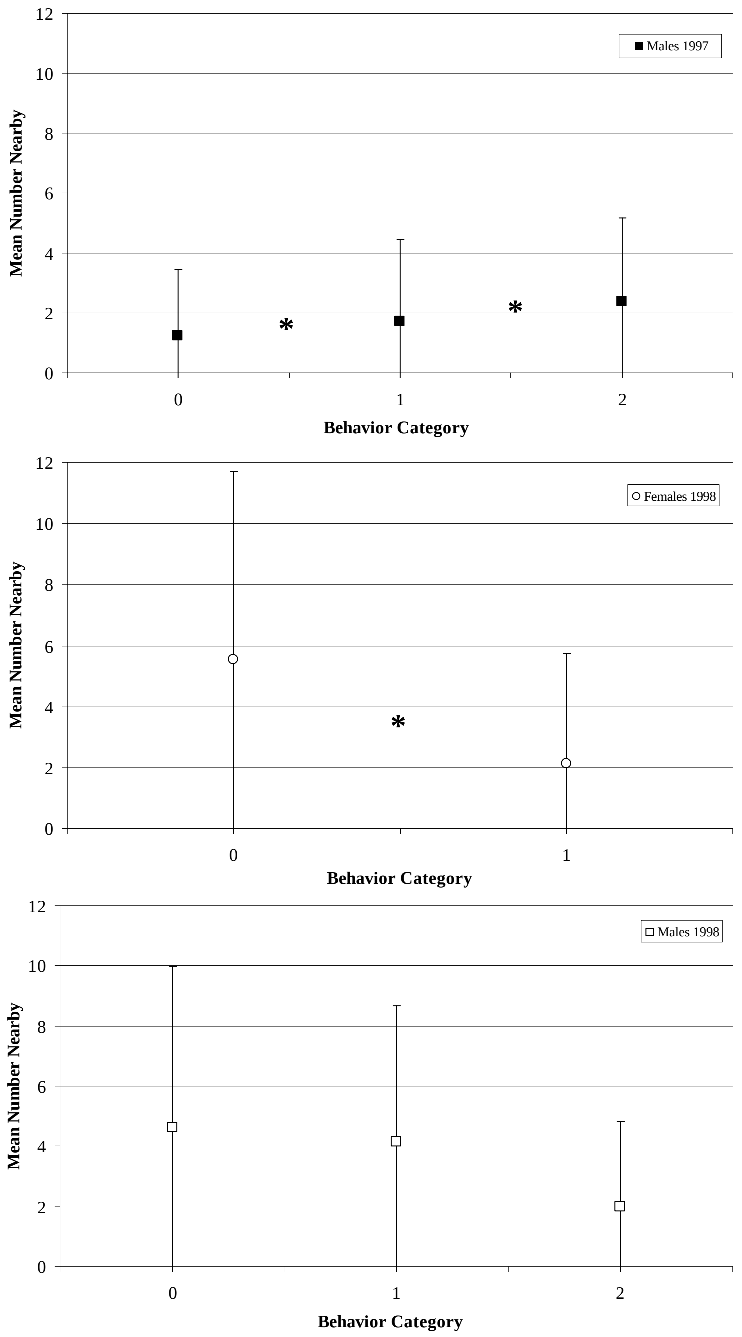


Figure 62. Mean ± SD of individuals within 1 m (female sized in 1997 and all in 1998) of territorial males or marked females in 1997 and 1998. Rest positions were categorized as 0, copulation attempts or activity (Cop and SW) were categorized as 2, and all other behaviors were grouped under category 1. Statistical significance is indicated with an asterisk (*).

Physical Measurement Data

Physical measurement data were entered into a spreadsheet (Microsoft® Excel 2000) and then sorted, tabulated, and analyzed with SPSS for Windows 11.0.0 in order to identify statistically significant differences between groups. All original data are included with this document on a CD/DVD for restructuring and further analysis at the reader’s discretion.

Population Means

Comparison of sampled population means for three categories of iguanas is presented in Table 3. The islet was visited three times; however, not all measurements were taken during each visit. The following general observations have been made based upon information provided in Table 3:

Males

With the exception of the mean number of ticks counted between 1997 and later in 1997 (labeled “mid 1997”) all measured means were significantly different from each other. This could either mean that the individuals that were measured changed significantly or that the type of individuals present during each period changed significantly. As will be seen later in Table 4 many significant changes in individuals occurred, but not enough or in the direction necessary to take into account all of the significant differences observed in males marked as territorial at each point in time, so it is concluded that males that were marked as territorial and remained on the islet beyond the reproductive season had significantly different characteristics compared to those that spent most of their time along the coast of Isla Santa Cruz. It is possible that food was less available around Caamaño compared to sites frequented by males that swam over during the reproductive season, so remaining males were limited in size for that reason. Exact cause for the distinction between the two groups is not known.

For all measurements of size (SVL, TL, HW, WT, LSN, HLL, LBS) the mean calculated for the 1998 reproductive season was significantly larger than that of the 1997 reproductive season. Mean number of ticks was significantly higher while mean number of mites was significantly lower in the 1998 season. Ticks survive better under humid conditions, which are prevalent during ENSO periods. Perhaps such a change enabled increased tick survival and led to greater numbers per individual. Influences on mite survival are not known. Measurements taken later in 1997 indicated males remaining on the islet weighed significantly less than males had during the earlier reproductive season and had significantly fewer mites, however, the difference in mean number of ticks between the two periods was not statistically significant.

Females

In contrast to the means obtained for territorial males marked females showed significant decreases in many measures of size (SVL, HW, WT) when the 1997 and 1998 reproductive seasons are compared. Additionally, mean number of ticks increased significantly between 1997 and 1998. For the three characteristics measured later in 1997 (WT, Ticks, Mites) significant differences were identified. Weight decreased significantly between 1997 and mid 1997 as well as between mid 1997 and 1998. Mean number of ticks increased significantly between 1997 and mid 1997 while mean recorded value for mites decreased significantly between 1997 and mid 1997.

Hatchlings

As might be expected of new organisms, every measure taken for hatchlings immediately after hatching when compared to later measures taken in 1998 showed statistical significance. Growth during the first year was substantial. Parasitic load changes were interesting. While mean number of ticks rose significantly mean recorded value for mites decreased significantly. The same type of decrease was also observed for males (between 1997 and 1998 reproductive seasons) while an increase was observed for females. The meaning of these observations is not clear.

Table 3. Changes in measured means over one year for selected physical traits in three segments of the marine iguana population on Caamaño are shown. One or more, as needed, asterisks (“*”) identify statistically significant (a=.05) differences between means as indicated by Tamhane’s T2 post hoc test.

			Males	Standard		**Females**	Standard		**Hatchlings**	Standard
Physical Trait/Measurement	Year	*n*	Mean	Deviation	*n*	Mean	Deviation	*n*	Mean	Deviation
SVL (snout-vent-length)	1997	236	*405.36	35.866	826	*273.42	25.385			
(mm)	mid 1997							488	*108.49	14.185
	1998	30	*432.23	33.518	50	*257.14	13.571	166	*132.93	6.722
TL (tail)	1997	235	*549.12	57.275	826	367.69	34.394			
(mm)	mid 1997							488	*148.01	8.577
	1998	30	*618.90	59.771	50	358.56	27.436	166	*181.73	12.336
HW (head width)	1997	236	*600.46	55.840	826	*371.43	28.568			
(.1 mm)	mid 1997							488	*168.49	15.183
	1998	30	*658.67	53.591	50	*360.02	17.635	166	*199.40	7.661
WT (weight)	1997	234	*,***3190.36	825.553	825	*,***847.97	221.976			
(g)	mid 1997	39	*,**1959.49	345.649	60	*,**710.25	122.182	489	*54.62	7.873
	1998	30	**,***3878.33	1030.687	50	**,***566.10	116.239	166	*100.93	22.659
LSN (longest spine on neck)	1997	236	*247.65	31.953	826	147.30	49.146			
(.1 mm)	mid 1997							489	*16.84	8.274
	1998	30	*270.23	37.804	50	135.22	23.330	166	*31.59	7.651
HLL (hind leg length)	1997	235	*196.44	20.155	826	132.10	9.793			
(mm)	mid 1997							488	*57.77	2.349
	1998	30	*217.60	23.847	50	130.66	7.577	166	*67.27	3.047
Ticks	1997	236	*3.69	5.610	826	*,***.83	2.025			
(total ticks)	mid 1997	39	**4.51	5.698	60	*3.73	2.648	489	*4.41	12.263
	1998	30	*,**9.17	6.828	50	***5.48	5.471	166	*15.66	16.637
Mites	1997	234	*,***1.40	.949	826	*.97	.770			
(relative #, 0-3)	mid 1997	39	*.51	.644	60	*,**.72	.715	489	*.50	.790
	1998	30	***.70	.837	50	**1.46	1.705	166	*.31	.602
LBS (longest back spine)	1997	235	*203.41	32.082						
(.1 mm)	mid 1997									
	1998	30	*227.23	40.973						

Paired Measurements

The following three tables (Table 4 - Table 6) contain means calculated from the same individuals that were measured multiple times, so they are paired means. Data recorded later in 1997 (labeled “mid 1997”) indicate for both males (Table 4) and females (Table 5) that all recorded measurements showed significant change between first and second measures during the year. Weight values went down, tick values went up, and mite values went down.

When the same individuals’ measures are compared between 1997 and 1998 reproductive seasons many traits followed similar patterns in the groups of males (Table 4) and females (Table 5), but there were some notable differences as well. SVL was significantly lower in both groups while an increase in TL was only significant for males. Change in HW was not significant for either group. Decrease in WT for both males and females was significant. LSN for males appeared to increase (although not significantly) at the same time that mean female LSN decreased significantly. Both groups showed an increase in HLL, but the mean of female measurements was the only one to show significant change. The same held true for mean number of ticks. Both showed increases while significant increase was only detectable in females. Change in mean recorded mites value decreased significantly for males while the value increased, however not significantly, for females. LBS was only recorded for males and was found to have increased significantly in individuals observed originally in 1997 and later in 1998.

Comparison of measurements for hatchlings observed both in 1997 and in 1998 (Table 6) indicates, as might be expected in new organisms, that changes in all characteristics were significant. All physical size measurements (SVL, TL, HW, WT, LSN, and HLL) increased significantly. Mean number of ticks increased while mean recorded value for mite infestation decreased.

Table 4. Changes in measured means over one year for selected physical traits in paired sets of male marine iguana data on Caamaño are shown. Asterisks ("*") identify statistically significant (a=.05) differences as indicated by two-tailed paired-samples *t* test.

Physical Trait/ Measurement	*n*	**1997** Mean	Standard Deviation	**mid 1997** Mean	Standard Deviation	*n*	**1997** Mean	Standard Deviation	**1998** Mean	Standard Deviation
SVL (snout-vent-length) (mm)						22	*438.59	36.062	*428.59	33.879
TL (tail) (mm)						21	*590.43	51.013	*603.00	59.270
HW (head width) (.1 mm)						22	654.73	51.836	655.86	53.354
WT (weight) (g)	38	*2220.79	430.257	*1941.32	330.872	22	*4086.82	977.652	*3778.41	995.714
LSN (longest spine on neck) (.1 mm)						22	262.18	31.832	267.77	39.194
HLL (hind leg length) (mm)						22	211.59	23.744	216.32	26.050
Ticks (total ticks)	38	*2.16	1.794	*4.61	5.745	22	6.14	9.306	8.64	6.083
Mites (relative #, 0-3)	38	*1.68	.873	*.50	.647	22	*1.27	.883	*.73	.883
LBS (longest back spine) (.1 mm)						22	*220.77	40.011	*230.32	42.166

Table 5. Changes in measured means over one year for selected physical traits in paired sets of female marine iguana data on Caamaño are shown. Asterisks (“*”) identify statistically significant (a=.05) differences as indicated by two-tailed paired-samples *t* test.

Physical Trait/ Measurement	*n*	**1997** Mean	Standard Deviation	**mid 1997** Mean	Standard Deviation	*n*	**1997** Mean	Standard Deviation	**1998** Mean	Standard Deviation
SVL (snout-vent-length) (mm)						50	*263.56	15.884	*257.14	13.571
TL (tail) (mm)						50	357.12	25.639	358.56	27.436
HW (head width) (.1 mm)						50	361.00	17.977	360.02	17.635
WT (weight) (g)	60	*832.25	178.120	*710.25	122.182	50	*790.30	163.415	*566.10	116.239
LSN (longest spine on neck) (.1 mm)						50	*142.54	24.506	*135.22	23.330
HLL (hind leg length) (mm)						50	*129.02	8.085	*130.66	7.577
Ticks (total ticks)	60	*.80	.898	*3.73	2.648	50	*.60	.756	*5.48	5.471
Mites (relative #, 0-3)	60	*1.08	.850	*.72	.715	50	1.00	.904	1.46	1.705

Table 6. Changes in measured means over one year for selected physical traits in paired sets of hatchling marine iguana data on Caamaño are shown. Asterisks ("*") identify statistically significant (a=.05) differences as indicated by two-tailed paired-samples *t* test.

Physical Trait/ Measurement	*n*	**mid 1997** Mean	Standard Deviation	**1998** Mean	Standard Deviation
SVL (snout-vent-length) (mm)	148	*108.72	4.052	*133.34	6.533
TL (tail) (mm)	148	*149.91	7.891	*182.74	11.535
HW (head width) (.1 mm)	148	*169.22	6.986	*199.86	7.382
WT (weight) (g)	148	*56.44	6.916	*102.33	22.219
LSN (longest spine on neck) (.1 mm)	148	*16.52	2.727	*32.15	7.309
HLL (hind leg length) (mm)	148	*58.27	1.968	*67.48	2.915
Ticks (total ticks)	148	*2.70	6.248	*16.04	17.152
Mites (relative #, 0-3)	148	*.53	.795	*.30	.567

Physical Traits and Later Observation / Survival

The next two tables (Table 7 for males and females and Table 8 for hatchlings) show statistical comparison data for the three major groups (males, females, hatchlings) when individuals initially captured, marked, and measured were grouped by the last date of their observation. All measures used are the original ones taken (during the 1997 reproductive season for males and females and upon initial capture after hatching for hatchlings). Using the information in the tables it is implied that the characteristics of those present at initial observation that survived longest, and were significantly different from the characteristics of others that were not observed later, are of survival benefit. One note needs to be made for males. Males observed later in 1997 (“mid 1997”) likely remained on the islet year-round, while other males returned to the shores of Isla Santa Cruz. Males present in 1998 primarily represent those that returned to Caamaño and not necessarily which within the initial group of males from 1997 actually survived.

Prior to closer examination of the results for each of the three main groups (males, females, hatchlings) provided in the tables it is interesting to note some general trends. Males and hatchlings that were observed in 1998 were larger than those that were last seen at initial measurement. The opposite was true for females. Females that were observed in 1998 were among the smaller individuals observed initially in 1997. Calculated standard deviation appears to follow some patterns too. For most measurements male standard deviation (SD) was the same or higher in those that were observed in 1998 when compared to those last seen in 1997, while for females and hatchlings those observed latest (having survived the longest) generally were composed of a group that had lower SD values in initial measurements.

Males

Values listed for males (Table 7) require more background information than females and hatchlings for valid interpretation. While females and hatchlings remain on the islet year-round many males present during each reproductive season are away most of the year. After the 1997 reproductive season many were observed along the shoreline of Isla Santa Cruz. Males remaining on Caamaño throughout the year are generally smaller than other males marked as territorial. Both Table 3 (page 109) and Table 7 show this, however while Table 3 shows measurements taken later in 1997 Table 7 shows initial measurements of those found later in 1997 (but are limited to the ones that were last seen at that time later in 1997, so those that remained and were also observed in 1998 are not included in the calculated means for mid 1997) and in 1998 on the islet. The table was prepared in order to evaluate possible attributes leading to higher survival (or increased probability of return).

For all of the physical size measurements (SVL, TL, HW, WT, LSN, HLL, LBS) of males that were observed both in 1997 and 1998 their 1997 measured means were larger than those that were only seen during the 1997 reproductive season. However, while all means were larger for those present in the 1998 group there was no statistically significant difference between the means of the two groups for measures of LSN, HLL, or LBS. For all measures, except ticks and mites, the means of both those only seen initially in 1997 and those observed in 1998 were statistically different from those last seen in “mid 1997.” The number of ticks found on those that remained on the islet after the 1997 reproductive season and were last seen during the later period was significantly lower than those last seen during the reproductive period of 1997, but not significantly lower (due to large SD) than those that returned in 1998. There was no significant difference in mean recorded mite infestation level between groups.

Females

Attributes leading to survival in females were quite clear, especially since at later sampling points ("mid 1997" and 1998) only part of those present were sampled. However, using the small samples obtained (50-57 individuals) statistically significant differences in initial body characteristics measured in 1997 were observed in those that survived to later points in time. The trend was clear. While not all means were significantly different, individuals with small measurements survived longer. Measures that this did not consistently hold true for (where the mean of those last observed at a particular point did not decrease for each later point) include HW and LSN. Infestation with ticks or mites did not appear to significantly affect survival, although those that survived longest did have a lower number of ticks in 1997.

Hatchlings

As mentioned earlier, larger hatchlings survived longer (Table 8). This held true for each measure of size except for LSN, although the only measures that showed significance were TL, WT, and HLL. Those that survived until 1998 initially had significantly fewer ticks. Additionally, those that survived until 1998 also as a group had a lower mean sex probe measurement, indicating that more females likely survived than males (although the difference between the means was not significant). Within the group that survived until 1998 mean time spent running in the physical test was higher, distance traveled was longer, and velocity was lower (though none of these measures differed significantly).

Table 7. Comparison of selected characteristics from initial measurements of male and female marine iguanas in 1997 on Caamaño, grouped by their last live observation date. One or more, as needed, asterisks (“*”) identify statistically significant (a=.05) differences between means as indicated by Tamhane’s T2 post hoc test.

Physical Trait/	Last		**Males**	Standard		**Females**	Standard
Measurement	Observed	*n*	Mean	Deviation	*n*	Mean	Deviation
SVL (snout-vent-length)	1997	177	*,***410.03	30.795	719	*274.08	25.800
(mm)	mid 1997	37	*,**363.27	19.991	57	**273.86	25.419
	1998	22	**,***438.59	36.062	50	*,**263.56	15.884
TL (tail)	1997	177	*,***557.95	51.059	719	*368.47	35.067
(mm)	mid 1997	37	*,**483.46	36.361	57	367.09	31.271
	1998	21	**,***590.43	51.013	50	*357.12	25.639
HW (head width)	1997	177	*,***608.75	46.796	719	*372.03	29.314
(.1 mm)	mid 1997	37	*,**528.54	25.051	57	**373.04	24.858
	1998	22	**,***654.73	51.836	50	*,**361.00	17.977
WT (weight)	1997	175	*,***3284.03	667.266	718	*852.66	228.447
(g)	mid 1997	37	*,**2214.32	434.316	57	839.39	173.584
	1998	22	**,***4086.82	977.652	50	*790.30	163.415
LSN (longest spine on neck)	1997	177	*248.50	31.726	719	148.13	51.798
(.1 mm)	mid 1997	37	*,**234.92	29.218	57	140.96	24.229
	1998	22	**262.18	31.832	50	142.54	24.506
HLL (hind leg length)	1997	176	*199.09	17.897	719	*132.31	9.933
(mm)	mid 1997	37	*,**174.84	10.018	57	132.14	9.029
	1998	22	**211.59	23.744	50	*129.02	8.085
Ticks	1997	177	*3.70	5.457	719	.85	2.146
(total ticks)	mid 1997	37	*2.16	1.818	57	.82	.909
	1998	22	6.14	9.306	50	.60	.756
Mites	1997	175	1.35	.964	719	.96	.757
(relative #, 0-3)	mid 1997	37	1.70	.878	57	1.07	.821
	1998	22	1.27	.883	50	1.00	.904
LBS (longest back spine)	1997	176	*205.22	30.852			
(.1 mm)	mid 1997	37	*,**184.46	23.660			
	1998	22	**220.77	40.011			

Table 8. Comparison of selected characteristics from initial measurements of hatchlings taken immediately after hatching in mid 1997 on Caamaño, grouped by their last live observation date. Asterisks (“*”) identify statistically significant (a=.05) differences between means.

Physical Trait/ Measurement	Last Observed	**Hatchlings**		
		n	Mean	Standard Deviation
SVL (snout-vent-length)	mid 1997	340	108.39	16.790
(mm)	1998	148	108.72	4.052
TL (tail)	mid 1997	340	*147.19	8.742
(mm)	1998	148	*149.91	7.891
HW (head width)	mid 1997	340	168.17	17.597
(.1 mm)	1998	148	169.22	6.986
WT (weight)	mid 1997	341	*53.82	8.137
(g)	1998	148	*56.44	6.916
LSN (longest spine on neck)	mid 1997	341	16.98	9.746
(.1 mm)	1998	148	16.52	2.727
HLL (hind leg length)	mid 1997	340	*57.56	2.469
(mm)	1998	148	*58.27	1.968
Ticks	mid 1997	341	*5.16	14.040
(total ticks)	1998	148	*2.70	6.248
Mites	mid 1997	341	.49	.788
(relative #, 0-3)	1998	148	.53	.795
Sex (sex probe depth)	mid 1997	339	72.59	33.366
(.1 mm)	1998	147	69.22	33.246
Temp	mid 1997	291	31.513	2.2032
(°C)	1998	126	31.306	2.4232
Speed/Time	mid 1997	296	2.5258	3.09753
(sec)	1998	126	2.7825	2.31972
Distance	mid 1997	294	2.299	2.0099
(m)	1998	126	2.490	2.1773
Velocity	mid 1997	293	.917926886908	.5036377666860
(m/sec)	1998	125	.842112220608	.3993364404308

Regression Analyses

Behavior Ranking

In order for an examination of the relationship between behavior and various physical measures or number of other iguanas nearby all behaviors exhibited within each group (marked males, females, and hatchlings) were given a value based upon the period of observation. Since there was no clear reason available for using any whole number ranking, each behavior was ranked according to the mean number of individuals found nearby as observations were recorded throughout the entire period. For example, based on all observations during 1997 males observed resting in position R1 had a mean number of .95 female sized iguanas nearby, while in 1998 the mean number of iguanas within 1 m of males observed in position R1 was 5.08. Each group (marked males, females, and hatchlings) during each period (1997 and 1998 reproductive seasons) had behaviors calculated and ranked separately. Rank values associated with specific behaviors during each observation period are shown in Figure 63. For clarity and accuracy it is important to note that during the 1997 reproductive period number nearby recorded for territorial males referred to number of “female sized” iguanas within 1 m, while in 1998 for all groups the number recorded refers to number of all other iguanas nearby, and not just to number of “female sized” iguanas. Identification as “female sized” was used because prior to full maturation males do not appear much different from females and can be identified with certainty only by cloacal probing. It is important to note that number of iguanas at any time not categorized as “female sized” was very small (estimated at 1-3% in 1998). For that reason comparison between 1997 and 1998 for territorial males is considered informative. Few marked hatchlings were observed so values for that group in 1998 are considered suspect.

One distinct and important observation concerning Figure 63 is the large difference in mean number near territorial males between the 1997 and 1998 reproductive seasons. For every behavior observed in both 1997 and 1998 the mean nearby was higher in 1998. For every behavior except Cop (copulating) and Wa (walking) the value calculated for 1998 was much higher.

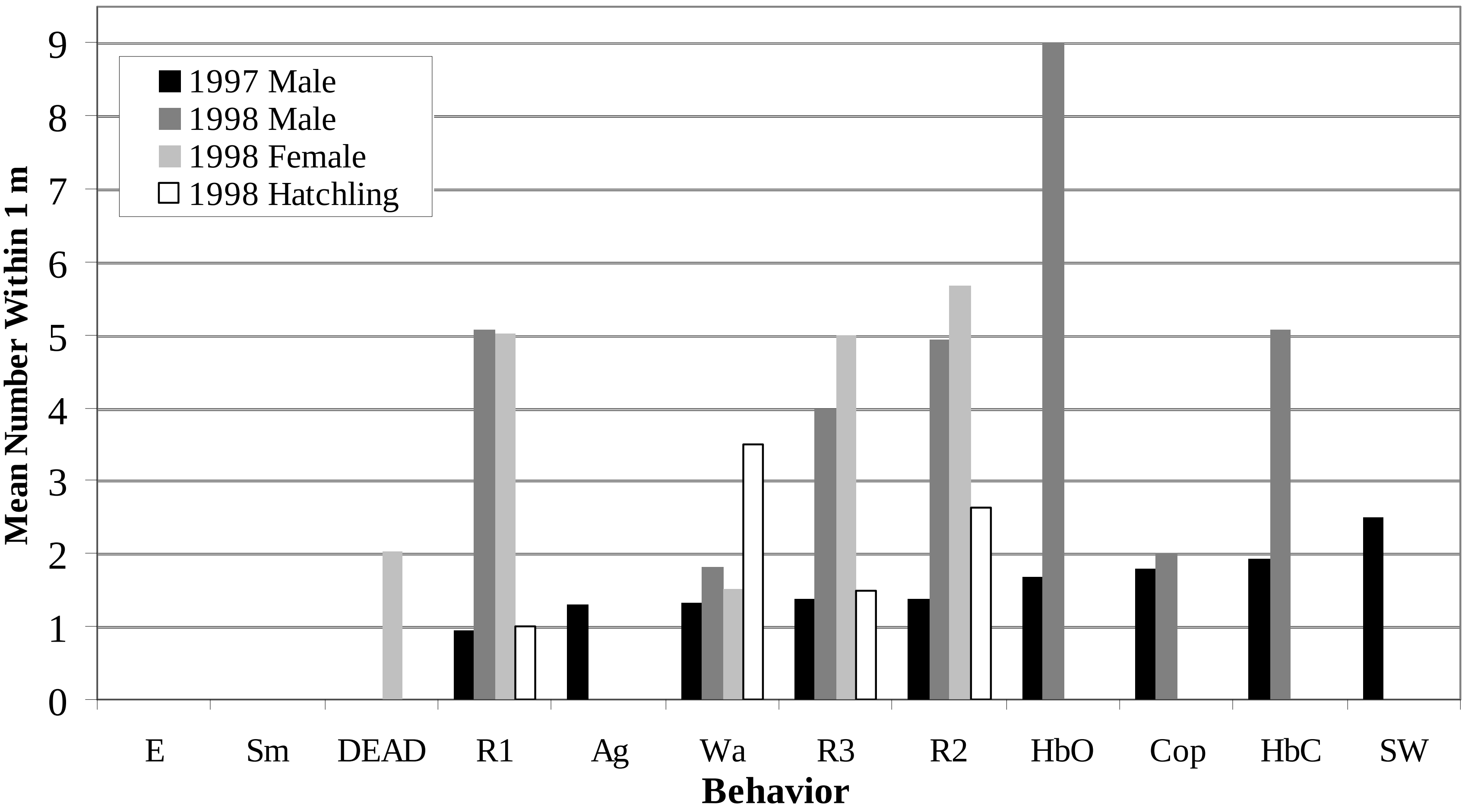


Figure 63. Mean number of individuals nearby associated with specific behaviors observed during the 1997 and 1998 reproductive seasons on Caamaño. 1997 values refer to mean number of “female sized” iguanas within 1 m while 1998 values refer to mean number of all iguanas nearby. Abbreviations for behaviors are as follows: Ag-agonistic, Cop-copulating, E-eating, HbC-headbobbing with closed mouth, HbO-headbobbing with open mouth, Sm-swimming, SW-side-walking, R1-resting mode 1, R2-resting mode 2, R3-resting mode 3, Wa-walking.

Predicting Behavior

Using assigned behavioral values for each group and period (which were based upon mean number of individuals nearby [see Figure 63 on page 123 and preceding explanation]) a mean behavioral value was computed for each individual using all available observation data. Once seasonal mean individual behavior values were produced, other measures were examined to determine whether they might be used to predict mean behavioral values calculated from all individual observations during a reproductive season. The measures taken together and used to most accurately predict mean behavior for various groups are listed below along with associated R^2 and adjusted R^2 values. See list of abbreviations (page xvi) and detailed descriptions of measures taken (beginning on page 11) where needed.

Because of the apparent circularity of using mean number of females or individuals nearby to predict mean seasonal behavior (which was calculated using values based upon number nearby) three equations are provided. First, an equation (labeled "1st") using all measures available (that had highest predictive value) is provided. It is followed by an equation (labeled "2nd") using only measures other than number nearby or behavior. Finally, a third equation (labeled "3rd") using only the most predictive single measure other than behavior or number nearby is given. Each equation was chosen using the fewest number of measures possible in order to achieve the highest adjusted R^2 value. Method of variable selection was trial and error with the aid of level of significance listed for each in SPSS output, either for single or multiple variables. This method produced equations with greater predictive value than any automated selection process available in SPSS.

1997 Territorial Males (1st) – R^2 = .492, adjusted R^2 = .474

Mean Behavior = Mean Females Nearby(4.942×10^{-2}) + SVL(9.245×10^{-4}) + HW(8.076×10^{-4}) + Ticks(1.567×10^{-3}) + LSN(2.227×10^{-4}) + Mites(8.657×10^{-3}) - WT(3.84×10^{-5}) - TL(2.12×10^{-4}) + .598

1997 Territorial Males (2nd) – R^2 = .327, adjusted R^2 = .315

Mean Behavior = HW(8.803×10^{-4}) + SVL(7.095×10^{-4}) - TL(2.41×10^{-4}) - WT(6.35×10^{-6}) + .692

1997 Territorial Males (3rd) – R^2 = .308, adjusted R^2 = .305

Mean Behavior = HW(9.784×10^{-4}) + .768

1998 Territorial Males – No significant predictability.

1998 Females (1st) – R^2 = .413, adjusted R^2 = .360

Mean Behavior = Mean Iguanas Nearby(8.169×10^{-2}) - SVL(1.64×10^{-2}) - LSN(1.30×10^{-2}) + WT(1.777×10^{-3}) + 9.349

1998 Females (2nd) – R^2 = .362, adjusted R^2 = .319

Mean Behavior = WT(2.681×10^{-3}) - SVL(2.14×10^{-2}) - LSN(1.37×10^{-2}) + 10.633

1998 Females (3rd) – R^2 = .237, adjusted R^2 = .221

Mean Behavior = 6.845 - LSN(1.52×10^{-2})

1998 Hatchlings – No significant predictability.

Predicting Number Nearby

Using ranked behaviors for each group and period (see Figure 63 on page 123) a mean behavioral value was computed for each individual. This mean behavioral value was useful in predicting mean number of individuals nearby (within 1 m) throughout the reproductive season. However, when combined with other measures predictability of mean number nearby for a season was much greater. The measures taken together and used to most accurately predict mean number of individuals nearby for various groups are listed below along with associated R^2 and adjusted R^2 values. See list of abbreviations (page xvi) where needed.

Because of the apparent circularity of using behavior (which was calculated using values based upon number nearby) to predict mean number of females or individuals nearby three equations are provided. First, an equation (labeled "1st") using all measures available (that had highest predictive value) is provided. It is followed by an equation (labeled "2nd") using only measures other than number nearby or behavior. Finally, a third equation (labeled "3rd") using only the most predictive single measure other than behavior or number nearby is given. Each equation was chosen using the fewest number of measures possible in order to achieve the highest adjusted R^2 value. Method of variable selection was trial and error with the aid of level of significance listed for each in SPSS output, either for single or multiple variables. This method produced equations with greater predictive value than any automated selection process available in SPSS.

1997 Territorial Males (1st) – R^2 = .393, adjusted R^2 = .377

Mean Number of "Female Sized" Iguanas Nearby = Mean Behavior(4.686) - SVL(7.958×10^{-3}) - HW(4.055×10^{-3}) + WT(6.626×10^{-4}) + HLL(6.383×10^{-3}) - Ticks(2.571×10^{-2}) - 2.673

1997 Territorial Males (2nd) – R^2 = .221, adjusted R^2 = .208

Mean Number of "Female Sized" Iguanas Nearby = WT(5.88×10^{-4}) - SVL(5.15×10^{-3}) + HLL(6.393×10^{-3}) - Ticks(2.13×10^{-2}) + .322

1997 Territorial Males (3rd) – R^2 = .198, adjusted R^2 = .194

Mean Number of "Female Sized" Iguanas Nearby = WT(4.951×10^{-4}) - .295

1998 Territorial Males (1st) – R^2 = .467, adjusted R^2 = .315

Mean Number of Iguanas Nearby = Mean Behavior(3.888) + SVL(3.469×10^{-2}) + WT(1.315×10^{-3}) - LSN(1.80×10^{-2}) - HLL(5.19×10^{-2}) - Ticks(8.37×10^{-2}) - 17.090

1998 Territorial Males (2nd) – R^2 = .266, adjusted R^2 = .207

Mean Number of Iguanas Nearby = WT(1.237×10^{-3}) - Ticks(.111) + .223

1998 Territorial Males (3rd) – R^2 = .179, adjusted R^2 = .147

Mean Number of Iguanas Nearby = WT(1.057×10^{-3}) - .101

1998 Females (1st) – R^2 = .242, adjusted R^2 = .209

Mean Number of Iguanas Nearby = Mean Behavior(1.152) + WT(5.359×10^{-3}) - 3.660

1998 Females (2^{nd} and 3^{rd}) – $R^2 = .108$, adjusted $R^2 = .089$

Mean Number of Iguanas Nearby = WT(6.409×10^{-3}) + 1.273

1998 Hatchlings – No significant predictability.

Predicting Survival

Using measures for males and females taken in 1997 no single or combined measures significantly predicted survival (or more accurately "return" for males). Using data collected it was not possible to obtain any adjusted R^2 value even up to .01.

Hatchling survival was slightly predictable. The single most predictive character was WT. R^2 was calculated at .023 and adjusted R at .021 (the resulting equation being, Time Survived Beyond Hatching in 1997 = 1996.815 + WT(8.922×10^{-3}). Highest predictability of survival for one year was obtained using WT, TL, Ticks, and Day Hatched. Day Hatched was the sequential day since hatching started. R^2 was .046 while adjusted R^2 was .038, which is not particularly predictive. The full equation follows:

Time Survived Beyond Hatching in 1997 = 1996.541 + TL(4.042×10^{-3}) – Ticks(2.509×10^{-3}) – Day Hatched(6.579×10^{-3}) + WT(5.159×10^{-3})

Noting the relatively high predictive value of Day Hatched alone ($R^2 = .020$ and adjusted $R^2 = .018$) prediction of hatching day based upon measured characteristics was evaluated. The single greatest predictor of what day an individual hatched was Distance (see detailed descriptions of measures taken beginning on page 11). $R^2 = .207$, adjusted $R^2 = .205$, and the full equation is Day Hatched = 20.190 – Distance(1.678).

However, for greatest predictability all measures except for HW needed to be taken into account. This led to an R^2 value of .384 and adjusted R^2 value of .364. The full equation follows:

Day Hatched = 2396.268 – Distance(1.616) + Sex(1.620×10^{-2}) – SVL(3.244×10^{-2}) – TL(7.742×10^{-2}) – WT(.135) + LSN(5.364×10^{-2}) + HLL(.298) + Ticks(5.351×10^{-2}) + Mites(1.375) – Temp(.840) – Speed/Time(.253) + Velocity(2.904) – Last Year of Observation(1.176)

Microsatellite Molecular Marker Data

Primers Tested

Microsatellite primer sets evaluated for use are listed in Table 9. If submitted to GenBank locus name matches that listed in GenBank. Laboratory notes for primer sets developed do not include the “AC(caam)” prefix, which was added for GenBank listing in order to identify the source of the DNA as *Amblyrhynchus cristatus* from Caamaño. All GenBank listings available are provided in Appendix E (page 183). Primer set “2nd Am(GT)-2” was so labeled because primer sequences used were redesigned to replace originals used by the producer of the sequence information (Rassmann 1997a) that did not work.

Type and length of repeat, cloned DNA size (in base pairs), number of observed alleles or product characteristics, along with observed and expected heterozygosity levels are provided for evaluation and decision making concerning use of the primers in other work. Annealing temperature and magnesium chloride concentrations are provided (in order that results reported might be replicated by others).

Primer sets that appear to have potential if PCR methods or alternate primer sequences are further refined have “Poten.” listed in the column indicating number of alleles observed. Primer sets that produced multiple bands (generally five or more) in complex, mostly un-interpretable, patterns are labeled with “Multi.” If all bands observed in sampled individuals were the same “=” was added to the description of allele number. Tested primer sets that were monomorphic and did not have multiple easily identifiable alleles were not characterized further, since the intent was to find molecular markers that might be used in paternity testing.

Table 9. Microsatellite primer sets evaluated for usefulness in marine iguana paternity testing. Primer sets used to determine successful paternity identification probability have observed (H_O) and expected (H_E) heterozygosity values listed. Primer sequences with possible future potential have "Poten." listed under number of alleles observed, while primers producing multiple complex groups of alleles are identified with "Mult." "=" indicates that each test produced the listed number of exact same alleles for each individual.

Locus	Repeat Type	Primer Sequences (5´-3´)	Cloned Size (bp)	T_a (°C)	[$MgCl_2$] (mM)	No. Obs. Alleles	H_O	H_E	GenBank Accession No.
AC(caam)14	$(TG)_{11}$	TAAAACAACCTGAGCACCCTG CCGTGAAGACATTACAGACCC	106	56	2.07	19	.60345	.87826	AF452618
AC(caam)22	$(AC)_{11}$	AAATAGATGACCAAAATG CACCCACTTACAGAAAAA	163	50	2.75	6	.21053	.73824	AY035298
AC(caam)29	$CTGT(GT)_{13}(CTGT)_5$	GTCCCTACCCTTGCTCTAA AACTTGTCCCATCACTTTG	269	64	2.75	Poten.	--------	--------	AF297084
AC(caam)30	$(TGTT)_2(TG)_{12}$	AATGATAACAATGATGGACTT TATGATAGAAATAGGGAAAAA	289	50	2.75	10	.24138	.84498	AY035299
AC(caam)40	$(TG)_{12}$	CCTCTTTTCCTTCTCAATAG ACTCAACTTTACGGGGTCAG	144	50	2.75	Smear	--------	--------	AY035300
AC(caam)42	$(AC)_2A(AC)_1$… $(AC)_8AA(AC)_4$	CGATGATTTTGCTTAC ACTTAGGTTGATGTCC	155	58	2.75	7	.30189	.74771	AY035301
AC(caam)46	$(CA)_4$..$(CA)_7CT(CA)_4$ $CG(CA)_2TA(CA)_2$	GCACAGTCTTCCAACCACACAA TCCCATTCCCATTTTATCATTT	272	60	2.75	None	--------	--------	AY035302
AC(caam)50	$(CT)_4(GT)_{15}$	CCTCCAAAGTAACATT AGCATCTCTGAACGAA	181	54	2.75	7	.35000	.75658	AY035303
AC(caam)56	$TGTA(TG)_5$ $(CATG)_1TG(CATG)_2$	TTTTTGCTGTGTCTGGATTTTG ATGGTTTGGTAAGTGCTATGTC	280	59	1.5	1	--------	--------	-------------
AC(caam)60	$(TC)_{22}TTTCTGTC$ $(TCCT)_4TTCCT$	AATGTGCTGTTTTGATAAGA TTTCCACATTCAGATTATTC	193	50	2	Poten.	--------	--------	AY035304
AC(caam)66	$(T)_{16}G((GT)_6CT)_2$	AAACCCTCCATTTGTATTGAGTGC ACCTTCCCTCTCCCCTCTTTCA	148	50	2.07	1	--------	--------	AF452619
AC(caam)119/147	$(TG)_{11}$	GGAATCTTTGAACTGGTTTTGG GGTCAATTCTAAGAAGCCCACA	123	45	2.07	8	.56667	.79090	AF452620
AC(caam)196	$(AT)_7(GT)_{13}$	CTTGTGTTAGGATAGATAGAGAGAATC GTTGTGCTGTTTCATTTTCATC	174	50	2.07	14	.91304	.86670	AF452621

Locus	Repeat Type	Primer Sequences (5´-3´)	Cloned Size (bp)	T_a (°C)	[$MgCl_2$] (mM)	No. Obs. Alleles	H_O	H_E	GenBank Accession No.
2nd Am(GT)-2[R]	$(TG)_{15}$	CAAAGGGCATTATCATC AACAGAACCACCAAAGT	154	48	2.07	14	.93220	.87672	-------------
Am(GT)-4	$(TG)_{15}$…$(CA)_4(AG)_3$ …$(CTTCT)_3$	TTATGGATGAGCAATAC GTATATATGCCTTGTAG	211	46	2.07	10	.63158	.82937	-------------
Am(GT)-7	$(AC)_{14}$	TAAGTTTATCTCCTGCC TGTTACCAGCAATGTGA	126	36-53	2.66	1	--------	--------	-------------
Co-18A	$(CTAT)_n(CCAT)_n$	ATAATGCTCTGAAATGGTGTG ACTCATTCTGACATCCATAAT	---------	53	2.07	7	.66071	.83237	-------------
Co-18B	$(TCC)_nA(CCA)_5$	GCATGGAAAACTTGATTATAT TTCCCTGTTATCTTTGACATA	---------	53	2.07	4	.40000	.53095	-------------
Co10F11	$(TC)_{17}$	GCCAGTTACTGTGATTGGTTCCA GGAACACATGCACAGAAAGCAA	102	57	2.47	Mult.	--------	--------	AF159512
Co12E10	$(GT)_{13}$	GCAGGCTCGTATGGAAGAAG ACCGACTCATCGTGCTTAAA	146	66	2.07	Mult.	--------	--------	AF159515
EST2	$(TTTC)_{28}$	CAGTGAAACTTTGAGTGTGAAG CCTGAGCTAGCATGACTATTCT	233	59	3.98	Mult.	--------	--------	AF069697
EST4	$(AGAA)_{19}$	ATCCAATACTCTGGCTTCCTA GCAAAGGGATTTACTTCTAAG	170	59	2.75	2=	--------	--------	AF069699
CCF-2/102[M]	$(GT)_n$[?]	AGGGAGATGATTTGAGTAAC CTAGTCAGGTGCTTGGT	---------	56	2.75	Mult.=	--------	--------	-------------
CCF-4/70	---------------------------	CCTGAGGGCCCTGTACTTTC TGATTTAACCCCCACTGGTG	---------	56	2.75	13	.87719	.88123	-------------
CCF-6/37	$(GT)_n$	AGTCGCTGGTGGGATGTT AGGCAAAAATATCYCAAAAGCAG	---------	56	2.75	Mult.=	--------	--------	-------------
L780	$(GT)_n$	GTTCACTTCCCAAGAACAGTGCA CCTCCTCTGTAGCAGAATGTAT	---------	56	2.75	1	--------	--------	-------------

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[R] Sequences and primer sets (except for re-analyzed primer set 2nd Am(GT)-2) were received through lab personal communication with Kornelia Rassmann concerning her Ph.D. work with *Amblyrhynchus* and *Conolophus* in the Galápagos.

[M] Primer sequences received through lab personal communication with Catherine Malone concerning her Ph.D. work with *Cyclura* and *Iguana* in the Caribbean.

[?] Sequences identified by screening with $(GT)_{18}$. Actual sequences not provided.

Allele Heterozygosity

Observed and expected heterozygosity for each of the 12 primer sets evaluated for use in paternity testing are provided in Table 10. Heterozygosity values that were previously recorded (Rassmann *et al.* 1997a) are also provided for primer sets with available information. An additional primer set (Am(GT)7) was tested thoroughly and found to produce only a single allele, which was quite different from the heterozygosity previously reported by Rassmann *et al.* (1997a).

Table 10 contains information on probability of alleles being in Hardy-Weinberg equilibrium as well. Results for all but two of the primer sets indicated probability of equilibrium as extremely remote. Primer sets in possible equilibrium were (1) set Am(GT)7, with a single allele found in all individuals – obviously in perfect equilibrium and (2) primer set Co-18A.

On the table's right side a grid indicating probability of linkage disequilibrium between 12 primer sets is provided. Numbers across the top match the order of the named primer sets found on the left side. However interpretation of the positive or negative indicators of significant disequilibrium must be done with the fact in mind that the test implemented in Arlequin 2.000 (Schneider *et al.* 2000) assumes Hardy-Weinberg equilibrium of genotypes (which is the case for few of the primer sets examined). Because linkage disequilibrium is a measure of deviation of observed gametic frequencies from those expected if alleles at different loci were chosen at random it is easy to see why having allele frequencies that are not in Hardy-Weinberg equilibrium would significantly affect calculation (and accuracy) of linkage disequilibrium values.

Table 10. Comparison of observed and expected microsatellite allele heterozygosity between two studies of marine iguanas on Caamaño, Hardy-Weinberg equilibrium test, and linkage disequilibrium[0] analysis within the population sample.

Microsatellite loci	*n*	Prev.[1] *n*	*A*	Prev.[1] *A*	H_O	Prev.[1] H_O	H_E	Prev.[1] H_E	H-W P value[2]	1	2	3	4	5	6	7	8	9	10	11	12
AC(caam)22	57		6		0.21053		0.73824		0.00000	*	+	+	+	-	-	-	+	-	+	-	+
AC(caam)30	58		10		0.24138		0.84498		0.00000	+	*	+	+	-	-	-	+	-	+	-	-
CCF-4/70[3]	57		13		0.87719		0.88123		0.00000	+	+	*	-	-	+	-	+	+	-	-	-
AC(caam)42	53		7		0.30189		0.74771		0.00000	+	+	-	*	+	-	+	-	+	-	-	-
AC(caam)50	60		7		0.35000		0.75658		0.00000	-	-	-	+	*	-	-	-	-	-	-	+
Co-18A[4]	56		7		0.66071		0.83237		0.15826	-	-	+	-	-	*	-	-	-	-	-	-
Co-18B[4]	60		4		0.40000		0.53095		0.00352	-	-	-	+	-	-	*	-	-	-	-	-
Am(GT)4[4]	57	12	10	6	0.63158	0.58	0.82937	0.73	0.00000	+	+	+	-	-	-	-	*	-	+	+	-
AC(caam)14	58		19		0.60345		0.87826		0.00000	-	-	+	+	-	-	-	-	*	-	-	-
2nd Am(GT)2[4,5]	59	12	14	10	0.93220	0.75	0.87672	0.86	0.00000	+	+	-	-	-	-	-	+	-	*	-	+
AC(caam)119/147	60		8		0.56667		0.79090		0.00000	-	-	-	-	-	-	-	+	-	-	*	-
AC(caam)196	46		14		0.91304		0.86670		0.00006	+	-	-	-	+	-	-	-	-	+	-	*
Am(GT)7[4]	60	12	1	8	0.00000	0.92	0.00000	0.82	1.00000												

n = sample size, *A* = total number of different alleles observed, H_O = observed heterozygosity, H_E = expected heterozygosity

[0] + indicates loci are linked at the significance level of P < 0.05.

[1] Previous measured data taken from the same population sampled on Caamaño by Rassmann *et al.* [Rassmann, K., D. Tautz, F. Trillmich, and C. Gliddon. 1997. The microevolution of the Galápagos marine iguana *Amblyrhynchus cristatus* assessed by nuclear and mitochondrial genetic analyses. ***Mol. Ecol.*** 6: 437-452.]

[2] P value < 0.05 indicates conditions of Hardy-Weinberg equilibrium not met.

[3] Primer sequences originally designed for *Cyclura cychlura figginsi* obtained from C. L. Malone.

[4] DNA sequencing originally performed and primers designed by K. Rassmann.

[5] Primers re-designed from sequence provided by K. Rassmann.

Potential Paternity Testing Value

With information available from characterization of primer sets the simulation capability of CERVUS 2.0 (Marshall *et al.* 1998) was used to evaluate paternity assignment possibility. Simulation parameters were set to: cycles = 10,000, number of candidate parents = 1,100, proportion of candidate parents sampled = 1.000, proportion of loci typed = 1.000, proportion of loci mistyped = 0.010. Based upon the simulation, when one parent is known the estimated success rate using the 12 primer sets was 100% up through the strict (95%) confidence level. However, under the same conditions when neither parent was known success rate at the strict confidence level was 38% while success at the relaxed (80%) confidence level was 79%.

Allele Frequencies Associated With Physical Data

In an effort to determine whether territorial male iguanas from 1997 with differing physical characteristics might share similar alleles Arlequin 2.001 (Schneider *et al.* 2000) was used to compare allelic frequencies for iguanas grouped by all of the physical measurements taken. Cutoff points between group measures were determined by plotting out measurements and looking for “natural breaks.” Three tests available in Arlequin 2.001 for evaluating genetic distance were computed (F_{ST}, R_{ST}, and an Exact test).

In Table 11 a summary of results from running combinations of all tests is displayed. For each test a dark indicator (F, R, E) represents a P value < 0.05 while a light indicator with a “?” (?F, ?R, ?E) represents a P value < 0.10. The page following Table 11 contains information on the size categories that were used and how many individuals were in each group.

Every measurement taken appeared to have at least one microsatellite primer set that indicated some allele frequency distributions could be associated with size categories. Additionally, every microsatellite primer set appeared to produce allele frequency data able to distinguish size categories within at least one measurement type (HLL, HW, LBS, . . .). Because of these interesting observations a computer program was created in order to help evaluate whether strong selection followed by rapid population expansion might lead to the observed results.

Table 11. Identification of statistically significant allele frequency differences between groups of territorial males, using individual microsatellite loci and then all loci combined, based upon physical characteristics and year of observation. F_{ST}, R_{ST}, and Exact tests were performed using Arlequin 2.000. See listing below table for symbol and grouping information.

	119/147	14(2)	196	22	Am(GT)2	30	42	50	Am(GT)4	CCF-4/70	Co-18A	Co-18B	All
HLL (hind leg length)			F		?R, ?E	F,?R,?E	?E		?F	?F, ?E			
HW (head width)	?F							?R			F, R,?E	F, R	
LBS (longest back spine)		F, R, E	?F		?F, ?E	?E	F, R, E	?F, E	F	F		F,?R, E	F, R
Location (section around islet)			?E		?F, R, E		?F, ?E						R
LSN (longest spine on neck)		?F, E				F, R, E		?E	F, R, E		F, R, E		
Mites			E	?R	F, R			R	R		F	?F, E	
SVL (snout vent length)			F, R		R, ?E	?E	?R	R	E	F, E	F, R, E		
Ticks	?F,?R			F		?F, E	R		?F, R, E			?E	
TL (tail)	F,?R,?E	?E	?F		?R, E	F		R		F, ?E			
WT (weight)	F, ?E		F, R		?F, R, E	?R,?E		?R	?F, ?E	F,?R, E	?R		?R
Year (time present/observed)	F, ?E	E	?E	F, ?E	E		?R	?F, E			E	?F,?R	F

F = $P < 0.05$ detected by F_{ST} R = $P < 0.05$ detected by R_{ST} E = $P < 0.05$ detected by Exact test ? = $P < 0.10$

Measurement ranges used to group individuals for tests performed and summarized in Table 11. Value in parentheses indicates sample size.

HLL:
<185 (10)
185-230 (36)
>230 (12)

HW:
<580 (13)
580-675 (33)
>675 (12)

LBS:
<174 (4)
174-258 (49)
>258 (5)

Location:
B-F (16)
I-N (43)

LSN:
<220 (5)
220-280 (38)
280-295 (7)
>295 (8)

Mites:
0 (17)
1 (24)
2 (11)
3 (6)

SVL:
<395 (11)
395-418 (22)
419-449 (14)
>449 (11)

Ticks:
0-3 (29)
4-13 (24)
>13 (5)

TL:
<500 (8)
500-615 (37)
>615 (13)

WT:
<3050 (24)
3050-3750 (17)
>3750 (17)

Year:
'97 (52)
'98 (31)
'97 & '98 (23)
Only '97 (29)
Only '98 (8)
All (60)

A Program for Simulating Populations

Based upon the results observed, located in Table 11 (page 137), it appeared that every attribute identified could be used to divide up individuals into groups, and that subsequently some microsatellite marker with allele frequencies that differed significantly between groups could be found. An explanation for this observation was desired. One possible explanation was that frequent population fluctuations (“booms and crashes”) might produce the observed results. To examine this possibility a computer program was created to test out the idea.

Documentation for the computer program is located in Appendix H (page 216). Because the main purpose of the program’s creation was to test out the feasibility of a simple explanation only a few repetitions and sets of variables were examined. Due to the number of variables involved any comprehensive tests evaluating effect of each variable on results would need to be done in a systematic manner. However, since mere initial testing of the idea was the purpose here, no comprehensive experimentation was completed. Two similar sets of variables were used to examine the possible effect that frequent population “boom and crash” might have on microsatellite loci. The first set, listed in Figure 64, was used to represent a population that did not experience periodic crashes (some variables listed in the file are not used because of that) while the second set of variables, listed in Figure 65, was used to represent a population that experienced population crashes every seven cycles or “generations.” Analyses of diversity between groups within the population at different points in time were performed using Arlequin 2.001.

```
 0                     Microsatellite Mutation Rate
 2000                  Number of Generations to Process
 5000                  Starting Population Size
 .5                    Male/Female Ratio in Starting Population
 .5                    Proportion of Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Longer Product
 1.965                 Average Number of Offspring Produced by each Female
 .5                    Male/Female Ratio of Offspring at Birth
 .5                    Probability of Survival from Birth to Year 1
 .8                    Probability of Survival from Year 1 to Year 2
 .9                    Probability of Survival from Year 2 to Year 3
 .95                   Probability of Survival from Year 3 to Year 4
 .95                   Probability of Survival from Year 4 to Year 5
 .95                   Probability of Survival from Year 5 to Year 6
 .9                    Probability of Survival from Year 6 to Year 7
 .9                    Probability of Survival from Year 7 to Year 8
 .8                    Probability of Survival from Year 8 to Year 9
 .7                    Probability of Survival from Year 9 to Year 10
 .6                    Probability of Survival from Year 10 to Year 11
 .5                    Probability of Survival from Year 11 to Year 12
 .5                    Probability of Survival from Year 12 to Year 13
 .5                    Probability of Survival from Year 13 to Year 14
 .5                    Probability of Survival from Year 14 to Year 15
 .5                    Probability of Survival from Year 15 to Year 16
 .5                    Probability of Survival from Year 16 to Year 17
 .5                    Probability of Survival from Year 17 to Year 18
 .5                    Probability of Survival from Year 18 to Year 19
 .5                    Probability of Survival from Year 19 to Year 20
 0                     Probability of Successfully Reproducing at Year 1
 0                     Probability of Successfully Reproducing at Year 2
 0                     Probability of Successfully Reproducing at Year 3
 .3                    Probability of Successfully Reproducing at Year 4
 .4                    Probability of Successfully Reproducing at Year 5
 .6                    Probability of Successfully Reproducing at Year 6
 .6                    Probability of Successfully Reproducing at Year 7
 .6                    Probability of Successfully Reproducing at Year 8
 .6                    Probability of Successfully Reproducing at Year 9
 .6                    Probability of Successfully Reproducing at Year 10
 .5                    Probability of Successfully Reproducing at Year 11
 .5                    Probability of Successfully Reproducing at Year 12
 .4                    Probability of Successfully Reproducing at Year 13
 .4                    Probability of Successfully Reproducing at Year 14
 .3                    Probability of Successfully Reproducing at Year 15
 .3                    Probability of Successfully Reproducing at Year 16
 .2                    Probability of Successfully Reproducing at Year 17
 .2                    Probability of Successfully Reproducing at Year 18
 .1                    Probability of Successfully Reproducing at Year 19
 .1                    Probability of Successfully Reproducing at Year 20
 1                     Number of Microsatellite Loci
 1                     Number of Phenotypes
 7                     Number of Generations to Cycle Through Before Pausing for a Sorting Event
 8                     Maximum Number of Offspring a Female Can Possibly Have
 0                     Phenotypic Mutation Rate
 .5                    Proportion of Phenotypic Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Larger Phenotype
assigned               Phenotype Determination Method Used ('assigned' or 'blended')
 1                     Intensity of Each Sort Event
relative               Method of Calculating Sort Events ('absolute' or 'relative')
C:\WINDOWS\Desktop\Diversion (running)\Test Data\None\Crash-Boom(None1).div
None                   Type of Sort Event to Simulate ('None', 'Upper', 'Lower', 'Center', 'U&L', 'U/C/L', 'Random')
```

Figure 64. Variables used in simulation for no periodic population declines.

```
 0                     Microsatellite Mutation Rate
 2000                  Number of Generations to Process
 5000                  Starting Population Size
 .5                    Male/Female Ratio in Starting Population
 .5                    Proportion of Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Longer Product
 4                     Average Number of Offspring Produced by each Female
 .5                    Male/Female Ratio of Offspring at Birth
 .5                    Probability of Survival from Birth to Year 1
 .8                    Probability of Survival from Year 1 to Year 2
 .9                    Probability of Survival from Year 2 to Year 3
 .95                   Probability of Survival from Year 3 to Year 4
 .95                   Probability of Survival from Year 4 to Year 5
 .95                   Probability of Survival from Year 5 to Year 6
 .9                    Probability of Survival from Year 6 to Year 7
 .9                    Probability of Survival from Year 7 to Year 8
 .8                    Probability of Survival from Year 8 to Year 9
 .7                    Probability of Survival from Year 9 to Year 10
 .6                    Probability of Survival from Year 10 to Year 11
 .5                    Probability of Survival from Year 11 to Year 12
 .5                    Probability of Survival from Year 12 to Year 13
 .5                    Probability of Survival from Year 13 to Year 14
 .5                    Probability of Survival from Year 14 to Year 15
 .5                    Probability of Survival from Year 15 to Year 16
 .5                    Probability of Survival from Year 16 to Year 17
 .5                    Probability of Survival from Year 17 to Year 18
 .5                    Probability of Survival from Year 18 to Year 19
 .5                    Probability of Survival from Year 19 to Year 20
 0                     Probability of Successfully Reproducing at Year 1
 0                     Probability of Successfully Reproducing at Year 2
 0                     Probability of Successfully Reproducing at Year 3
 .3                    Probability of Successfully Reproducing at Year 4
 .4                    Probability of Successfully Reproducing at Year 5
 .6                    Probability of Successfully Reproducing at Year 6
 .6                    Probability of Successfully Reproducing at Year 7
 .6                    Probability of Successfully Reproducing at Year 8
 .6                    Probability of Successfully Reproducing at Year 9
 .6                    Probability of Successfully Reproducing at Year 10
 .5                    Probability of Successfully Reproducing at Year 11
 .5                    Probability of Successfully Reproducing at Year 12
 .4                    Probability of Successfully Reproducing at Year 13
 .4                    Probability of Successfully Reproducing at Year 14
 .3                    Probability of Successfully Reproducing at Year 15
 .3                    Probability of Successfully Reproducing at Year 16
 .2                    Probability of Successfully Reproducing at Year 17
 .2                    Probability of Successfully Reproducing at Year 18
 .1                    Probability of Successfully Reproducing at Year 19
 .1                    Probability of Successfully Reproducing at Year 20
 6                     Number of Microsatellite Loci
 1                     Number of Phenotypes
 7                     Number of Generations to Cycle Through Before Pausing for a Sorting Event
 8                     Maximum Number of Offspring a Female Can Possibly Have
 0                     Phenotypic Mutation Rate
 .5                    Proportion of Phenotypic Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Larger Phenotype
assigned               Phenotype Determination Method Used ('assigned' or 'blended')
 .5105                 Intensity of Each Sort Event
relative               Method of Calculating Sort Events ('absolute' or 'relative')
C:\WINDOWS\Desktop\Diversion (running)\Test Data\Random\Crash-Boom(Random).div
Random                 Type of Sort Event to Simulate ('None', 'Upper', 'Lower', 'Center', 'U&L', 'U/C/L', 'Random')
```

Figure 65. Variables used in simulation for periodic population declines due to “sort events.”

The original population was generated randomly and had phenotypes ranging from -10 through 10 and microsatellite allele values ranging from 90 through 110. Analysis of the original population indicated that there were no differences in allele frequencies between three groups divided up based upon phenotype value. Phenotype ranges were from -10 through -3, -2.9… through 2.9…, and 3 through 10.

Tests indicated that number of microsatellite loci used influenced how soon differences between phenotypic groups were observed. If few loci were used differences appeared much sooner than when many were used. Table 12 presents some general data concerning how many "generations" were required before analysis of microsatellite alleles indicated groups based upon phenotype could be distinguished from one another (when in fact they came from exactly the same population).

Table 12. Approximate number of cycles (or generations) necessary in computer simulations before allelic frequencies between groups based on phenotype are observed to differ significantly.

Microsatellite Loci	No Periodic Selection	Periodic Selection
1	˜ 1200	˜ 10
2	˜ 1500	˜ 100
3	˜ 1500	˜ 200
4	>2000	˜ 2000
6	>2000	>2000

Based upon these simulations it is quite easy to imagine the known frequent, and often regular, population fluctuations experienced by marine iguanas in the Galápagos affecting allele frequency patterns in the same way. For this reason only phenotypes associated with characteristic allelic frequencies using all 12 microsatellite loci examined are considered especially useful at this point. As indicated in Table 11 (page 137) there is the possibility that Longest Back Spine, Location (around the shoreline of the islet), and Year of presence during the reproductive season are associated with specific allele frequencies. Cause of any association is undetermined.

DISCUSSION

Data presented add to the pool of reported information concerning Galápagos marine iguanas (*Amblyrhynchus cristatus*). During three visits to the small islet of Caamaño morphological and behavioral data were collected for two reproductive seasons (1997 and 1998). Observations and measurements provide detailed information concerning behavior and survival during an El Niño-Southern Oscillation (ENSO) event. In addition to providing data concerning what attributes might influence survival (and therefore reproductive success) molecular markers were developed that are potentially useful in paternity analysis or other examinations of population structure. Much has been written about marine iguana behavior (Carpenter 1966, Dellinger 1996, Laurie 1990-1990b, Partecke *et al.* 2002, Trillmich 1983, Wikelski *et al.* 1994-2001) and some about use of molecular markers for population analyses (Rassmann *et al.* 1997-1997b). Most significantly added here is a large amount of behavioral observation and physical measurement data and development of multiple microsatellite markers making paternity analysis feasible, as well as suggested cautions when interpreting molecular data.

Initial comparison of differences between the two reproductive seasons is striking. While the number of unique males observed daily in 1997 was around 200 (see Figure 9 on page 39) in 1998 the number of unique territorial males seen each day was around 30 at its highest point (see Figure 14 on page 44). Survival of males could not be determined because almost all territorial males left the islet after each reproductive season. However, females are not known to leave the islet at any time, so knowing that 827 females were marked during the visit one year earlier it is conspicuous that no more than 130 individuals were observed during any given day in the 1998 reproductive season (see Figure 14 on page 44). Additional dead females (marked and unmarked) were seen daily during the 1998 observation period. For hatchlings the data are even more striking. Though 490 hatchlings were marked in 1997 no more than five (5) unique individuals were observed in any given day in 1998 (see Figure 14 on page 44).

Paired comparisons of mean weight taken during 1997 and 1998 for both males (Table 4, page 112) and females (Table 5, page 113) showed that weight for both groups had declined significantly. Snout-vent-length decreased significantly for both males and females, however other measures increased, notably tail and hind leg length. For two other paired means, head width and longest spine on the neck, size increased in males but decreased in females. Longest back spine on males also increased significantly. Whether or not absorption of bone led to change in size, as presumed by Wikelski and Thom (2000), is logical speculation (which hopefully leads to more detailed examination), however, shrinkage of all measures during lean conditions does not appear to be the case.

It is worth pointing out that at this same time of death and weight loss some individuals (females generally) were observed eating land plants (*Portulaca sp.*) quite regularly. This was previously noted in Table 2 of an article entitled, “Niche expansion, body size, and survival in Galápagos marine iguanas” (Wikelski and Wrege, 2000). Dining on or testing of several other categories of alternative nutrition listed in Table 2 of the article was also observed during completion of this work as well (specifically, sea lion and marine iguana feces, regurgitates of birds, and afterbirth of sea lions).

Classification of Galápagos marine iguana reproductive behavior has changed with time. Trillmich and Trillmich (1984) consider the mating systems of both the pinnipeds (Galápagos fur seals, *Arctocephalus galapagoensis*, and Galápagos sea lions, *Zalophus californianus wollebaeki*) and marine iguanas to be a result of convergent evolution of polygyny. At least some of the work they based their analysis on was done on Caamaño (Trillmich 1983). During the 1997 reproductive season there were four large male sea lions protecting territories along the shoreline. Defense was generally directed toward males; however, human encroachment elicited vigorous response as well. For both species (sea lions and marine iguanas) male activity on the islet was limited to the reproductive season. Further work (Wikelski 1996, Wikelski *et al.* 1996) has led to classification of marine iguanas as exhibiting lek behavior. However, it is generally accepted that differing populations, or even the same populations at different times or under different circumstances, may exhibit characteristics of multiple reproductive systems. Such flexibility and possibility of this appears evident at almost all times within the marine iguana population, where multiple mating strategies are evident, especially during greater competition. Males without permanent territories and their activities have been well described as they "harass" females (Trillmich and Trillmich 1984, Wikelski *et al.* 1996, Hayes *et al*. 2004).

Further refinement concerning what exactly is considered a "lek" has often been attempted. One broad view is expressed by Alexander (1975) where he states: "In both vertebrates and invertebrates, however, the *lek* concept is appropriately applied to actively aggregating male groups within which all or nearly all mating occurs." Using his broad definition he also introduced the "resource-based lek" concept. Here resources available at defended territorial sites were not used to eliminate the system as a lek. Males could easily group within an area of resources females needed and through their defense of area potentially limit the access of other males to females. This was deemed perfectly within the definition of a lek as long as females themselves were not herded or restricted in their movements. This last qualification appears to identify a significant difference between the behavior of male marine iguanas and male sea lions.

During the period of observations on Caamaño in 1997 (since due to the ongoing ENSO no long lasting territoriality of sea lions was observed in 1998) male sea lions were often seen to try to keep females from leaving the territory they controlled. At no time in either 1997 or 1998 were any territorial male marine iguanas observed to attempt limitation of female movement. All male interaction with females was in the form of headbobbing and/or sidewalking (chasing in the case of males not holding permanent territories) or biting of the neck in an attempt to immediately copulate.

Using the broadened lek definition of Alexander (1975), detailed observations and analyses of Wikelski (1996) and Partecke *et al.* (2002), and consensus of not narrowing definitions *too* much, so as to become irrelevant (Höglund and Alatalo 1995), it is abundantly clear that marine iguanas can indeed be characterized as exhibiting lek behavior, even if some resource(s) might currently (or in the future) be identified within male territories. Males clearly gather within limited areas during the reproductive season and based upon observations up to this point the vast majority of all copulations occur among males within the bounds of long lasting territories.

Comparison of territorial male behavior between 1997 and 1998 shows marked differences. During 1997 aggressive and reproductive behaviors (Ag, Hb, HbO, SW, and Cop) were observed over an extended period (see Figure 38, on page 76). But in 1998, when there were few males, they displayed little, if any, aggressive or reproductive behavior, and what was observed was seen on only a few initial days during the observation period (see Figure 41, on page 79). It might be argued that observations in 1998 were started at a later date (January 19 vs. January 10 in 1997); however, persistence of the behaviors in 1997 lasted well beyond the date observations began in 1998.

Examination of numbers of iguanas associated with territorial males (based on counts of iguanas within 1 m, female sized in 1997 and all in 1998) for the two seasons is of value. In 1997 (see Figure 44, on page 83) between 40-50% of all observations of territorial males found no females located within 1 m. However, in 1998 (see Figure 47, on page 86) observations of territorial males with no iguanas within 1 m was reduced to between 15-30%. While it could be argued that there were two different criteria in effect in the count of number nearby (females sized in 1997 and all in 1998) there were almost no individuals (1-3%) in 1998 not considered “female sized.” In 1998 the 3-D charts look quite similar for territorial males and females (see Figure 47 and Figure 48, beginning on page 86).

Viewing numbers of iguanas associated with specific male behaviors between the two years is also informative. Figure 50 (page 93) shows data for territorial males in 1997 and Figure 56 (page 99) shows the same information for 1998. In 1997 relatively few iguanas were associated with males, no matter what behaviors were displayed, while in 1998 many more (three to four times as many) iguanas were found nearby for behaviors observed and comparable (the three rest states and Hb). However, even though more iguanas were found nearby territorial males in 1998 there were still fewer nearby than for the same behaviors observed in females (see Figure 58, on page 101).

Another view of the change in numbers of individuals associated with territorial males is provided in Figure 62 (page 105). Here behaviors have been grouped (rest as type 0, Cop and SW as type 2, and all others as type 1). Comparison of the trends for territorial males for both years provides interesting information and fodder for experimental suggestions. In 1997 the more aggressive activity was (toward females) the more females around. Differences between grouped behaviors were all statistically significant. However, in 1998, a year of wasting away and death, greater territorial male activity was associated with fewer females nearby. While differences were not statistically significant (likely because extremely few copulation behaviors were observed during the whole period in 1998) the general trend suggests important differences in male behavior and female site preference under altered conditions.

One simple explanation for these observations would be that male aggressive territorial and reproductive behavior is not particularly attractive to females, who are at most times primarily interested in sitting and digesting algae. Wikelski *et al.* (2001) report that extra female movement during the reproductive season greatly increases energy expenditure. More frequent disruptions from peaceful digestion of algae were tied to weight loss. Since territorial male behavior was more subdued in 1998 there was less impetus to move away. However, greater calm was still present among non-territorial animals (as indicated by the values for number nearby marked females).

But escape from male harassment is not always possible. As reported by Wikelski *et al.* (1996), male harassment is often greater outside male territories than it is inside, so females may in fact simply be pushed onto territories and accept the harassment there rather than roaming freely. Partecke *et al.* (2002) point out that males exhibit greater activity when territories are closely associated. This would explain the report by Wikelski *et al.* (1996) that number of females per male is higher on larger more dispersed territories where Partecke *et al.* (2002) report males are less active. Females would tend to stay where they are harassed least. While not “searching” for a male to mate with, quite clearly presented by Wikelski *et al.* (2001), females would likely serve themselves best by finding a male that protects a territory from other roving males and has as little time as possible to attend to females on his own territory. Experimental manipulations of territory size and female density could provide information necessary for further explanation of the reproductive system – what females prefer and how males react.

Comparison of measured means between visits (reproductive season in 1997, May/June 1997, and reproductive season in 1998) for three segments of the population provides data of interest (see Table 3, on page 109). While individual male weights decreased significantly between 1997 and 1998 (see Table 4, on page 112) mean weight of territorial males present in 1998 was greater than the mean in 1997. This indicates that only the largest (and presumably healthiest) males returned to the islet and defended territories. For females there is a steady decline in mean weight beginning with initial measurement in 1997. Since hatchlings were newly developing individuals, all size measures for those survivors captured in 1998 were significantly larger, as expected. However, at the same time standard deviation within the group of those that survived increased between years (Table 6, on page 114).

Examination of the standard deviation (SD) values on tables showing paired means for males, females, and hatchlings between time periods provide interesting insights (Table 4 - Table 6, beginning on page 112). Many of the measures for males showed increases in both mean and SD, but the opposite was true for most female measures (means declined along with SD). Measures that did increase with females were tail and hind leg length. Tail length increase could be explained by the observed “looseness” in iguanas of poor condition. A “loose” tail could easily have just been stretched more during measurement. However, significant increase in female hind leg length along with a corresponding decrease in SD seems to be consistent with at least some continued growth, even as snout-vent length and weight decreased. Growing hatchlings more closely mirrored male results, with measures increasing along with SD values.

Equivalent patterns of increased size and SD for both males and hatchlings would seem to indicate that growth was continuing in both groups, at least in survivors. Individuals were not becoming heavier, but they were continuing to grow. Females appeared to be starving to death, with larger individuals dying off first. With larger individuals eliminated mean for the group decreased along with SD. Preferential elimination of smaller and/or larger than average individuals might lead to reduced SD, but since means for the 1998 group as a whole, compared to 1997, also declined expiration of the largest individuals, as previously reported by Wikelski and Wrege (2000), is explanatory.

Using initial measurements for males and females, characteristics leading to either return (for males) or survival (for females and hatchlings) were identified (see Table 7 and Table 8, beginning on page 119). In general large males returned for the next reproductive season (even though they had lost weight between years, see Table 4 on page 112) and small females survived longest, while largest hatchlings at initial measurement lasted longest. Prediction of survival (or return) based upon physical characteristics was difficult or impossible (especially for males and females). However, the formula determined for hatchlings is found on page 128. Difficulty in finding a satisfactory model to predict return or survival is attributed to a wide range of animals of all sizes and shapes dying off or failing to return. Factors other than those measured appear to have had greater impact on return (failure to return) or survival (mortality). Which unmeasured factors might be influential could range from feeding location to learned behavior of some kind to complex (random) environmental interaction(s) undetectable at this time.

Microsatellite molecular markers were developed (enhanced and sped up using a secondary screening protocol that was developed, see Appendix B beginning on page 165) to enable paternity testing. However, the large total number of offspring and potential parents along with no way to reduce the number of possible parents for each hatchling made analysis unfeasible. Based upon the simulation ability of CERVUS 2.0 (Marshall *et al.* 1998), using the 12 most promising microsatellite markers would theoretically permit matching of all hatchlings with parents if one parent was known, however, if no parents were known only 38% of offspring and parents could be matched up accurately when the strict 95% confidence level was used. These results point out the value of, if at all possible, obtaining information on the identity of at least one parent. Attempts to track females and match up hatchlings with maternal genotype failed. Fences of plastic sheeting were setup around nest sites specific females were observed covering, however, no hatchlings were ever captured within those enclosures. Larger group enclosures were more successful, but because so many females laid eggs in the same areas (and enclosures were continually being knocked down by roaming sea lions) even those attempts to narrow females in the genetic pool failed. Enclosures did aid in capture of at least some hatchlings, but around half of all hatchlings were caught with fishing line nooses along the rocky shoreline during daily observation periods.

Although use of developed microsatellite markers for paternity analysis was not possible at this time, sequences were submitted to GenBank in order that others might use them for other purposes (such as population studies). Currently they are the only microsatellite sequences listed for marine iguanas and compose 3% of all microsatellite sequences listed for Squamata, 21% for Iguania, 23% for Iguanidae, and 70% for Iguaninae.

Examination of allele frequencies found within categories of physical measurement data for a sample of the territorial male population produced results stimulating many questions. Nearly every microsatellite locus (assumed to be neutral and not actively selected for or against) had some allele frequency that was associated with one or more physical measurements. This information is shown in Table 11 (page 137). Because of the high association of specific allele frequencies with physical attributes the possibility that frequent population fluctuations might produce such results was considered. In order to test out the idea a computer program was created to simulate populations. Variables included allowed multiple configurations to be tested. These are reported beginning on page 139. In short, use of few, 6 or less microsatellite loci (see Table 12 on page 142), with fluctuating population size (up to 90% mortality at times for marine iguanas [Wikelski and Wrege 2000]) could produce individual microsatellite marker allele frequency results similar to those observed in the Caamaño population sample within just a few years. However, use of all 12 microsatellite loci should provide reliable results. Simulation runs for 12 microsatellite loci could not be run long enough to detect apparent “linkage” of allele frequencies to physical characteristics. Additional extremely long runs may provide approximate number of cycles necessary for such “linkage” to appear when 12 loci are used, but it is assumed to be greater than 100,000 cycles. While 100,000 cycles is significantly less than the projected 10.5 million years since presumed divergence of Galápagos marine and land iguanas at constant evolutionary rate (Rassmann 1997), the variables used in the simulation did not include any mutation rate for either microsatellite alleles or physical attributes. Inclusion of mutations could further increase time/cycles necessary for any apparent “linkage” of allelic frequencies with phenotype(s) to occur.

For reasons just mentioned attributes that appear to have specific allele frequencies associated with them when all 12 microsatellite loci are used are considered important. Those specific attributes are (as shown under “All” in Table 11 on page 137) longest spine on the back, location (section around the islet), and year when the individual was observed present. Two of the identifying measures are not direct physical attributes (location and year). It seems quite possible that related individuals might be found at similar locations and that those males present and territorial during 1998 were more related to one another than those present in 1997. While these results certainly are not conclusive they point out possible future study options.

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APPENDICES

APPENDIX A – SOLUTIONS AND REAGENTS USED

30% Acrylamide:

29 g acrylamide

1 g *N*, *N'*-methylenebisacrylamide

dH_2O up to 100 ml

Heat to 37ºC to dissolve chemicals

Store at room temperature in total dark (covered in foil)

10% Ammonium Persulfate:

1 g ammonium persulfate

dH_2O up to 10 ml

Store at 4ºC (up to several weeks)

Ampicillin Solution:

100 mg/ml ampicillin

In distilled water sterile filtered through 0.22 micron filter

Store at 4ºC

Calcium Chloride Solution:

0.05 M $CaCl_2$

In distilled water sterile filtered through 0.22 micron filter

Denaturing Solution:

0.5 M NaOH

1.5 M NaCl

In distilled water

IPTG Solution:

100 mg/ml isopropyl-[beta]-D-thiogalactopyranoside

In distilled water

Store at -20ºC

Lithium Chloride Solution:

4 M LiCl

In distilled water

Sterile filtered through 0.22 micron filter

Lysis Buffer:

100 mM EDTA

10 mM Trizma-base (pH 7.5)

1% SDS

In distilled water

Neutralizing Solution:

1.5 M NaCl

0.5 M Tris-HCl

In distilled water

pH adjusted to 7.4

PCR Master Mix (standard – mixed on ice):

374.5 µl	dH_2O (distilled, autoclaved, filtered)
52.5 µl	$MgCl_2$ (25 mM stock solution sent with *Taq* from Promega)
63 µl	10x Buffer (comes with *Taq* in Buffer B from Promega)
63 µl	dNTP's (2.5 mM of each dNTP in Epicentre stock solution)
3.5 µl	Upper primer (250×10^{-12} mol stock solution)
3.5 µl	Lower primer (250×10^{-12} mol stock solution)
7 µl	*Taq* (in Buffer B from Promega)

Queen's Lysis Buffer:

0.1 M EDTA

0.1 M Trizma-base

2% SDS

Two or more volumes buffer to 1 volume blood

20x SSC:

3 M NaCl

0.3 M Sodium citrate

In distilled water

pH adjusted to 7.0

50x TAE (Tris acetate EDTA):

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Adjust volume to 1 l

TE (Tris-EDTA) Buffer:	Initial DNA Extraction and Storage:
0.01 M Tris-HCl	0.01 M Tris-HCl
0.001 M EDTA	0.2 mM Na_2EDTA
pH adjusted to 8.0	pH adjusted to 7.5
Sterile filtered through 0.22 micron filter	

Tris-HCl Buffer:

0.05 M Tris-HCl

pH adjusted to 7.2

Sterile filtered through 0.22 micron filter

Wash Buffers - Low / High Stringency:

Low 2x SSC / High 0.5x SSC

0.1% SDS Low / High

In distilled water

X-gal Solution:

20 mg/ml 5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside

In dimethylformamide

Store at -20ºC

APPENDIX B – PRINCIPLE BEHIND SECONDARY SCREENING

Figure 66 shows a graphical overview of the procedure developed and is followed by a detailed description of how bands were interpreted.

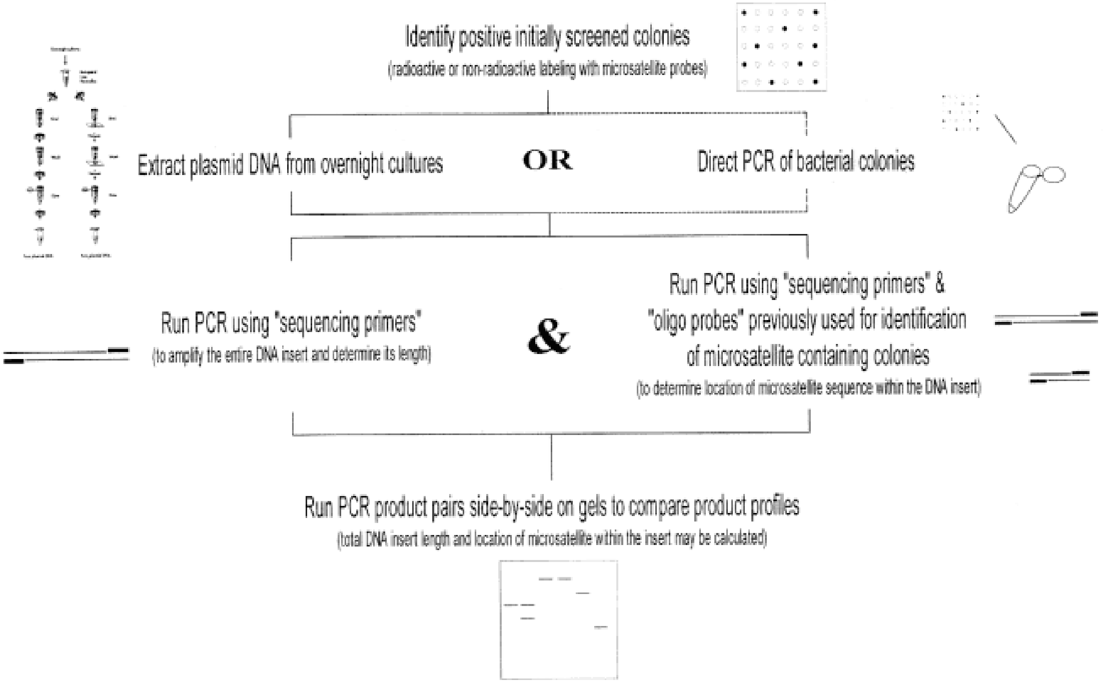
Identify positive initially screened colonies
(radioactive or non-radioactive labeling with microsatellite probes)
Extract plasmid DNA from overnight cultures
OR
Direct PCR of bacterial colonies
Run PCR using "sequencing primers"
(to amplify the entire DNA insert and determine its length)
&
Run PCR using "sequencing primers" & "oligo probes" previously used for identification of microsatellite containing colonies
(to determine location of microsatellite sequence within the DNA insert)
Run PCR product pairs side-by-side on gels to compare product profiles
(total DNA insert length and location of microsatellite within the insert may be calculated)

Figure 66. Overview of partial genomic library secondary screening methods.

Briefly, the process of gathering additional information about DNA inserted into the pUC19 plasmid in order to make a decision about what DNA was worth sending out to have sequenced involved three steps:

1. Select bacterial colonies that look promising from the hybridized membranes (those that have bound a lot of the "probes" containing the sequences of interest).

2. Amplify the inserted/cloned DNA using PCR primers (2 reactions, one with and one without the "probes" being used as primers) and run the products on a polyacrylamide gel.

3. Interpret the band patterns obtained for each pair of PCR products.

The selection of promising colonies, extraction of DNA, and PCR runs are described in Appendix G (page 215). Running PCR products on gels is described in principle in Appendix C (page 178) and with more detail in Appendix D (page 180) and in chapter 2 (page 26). The remainder of this appendix discusses the interpretation of gel banding patterns. Integral to the interpretation of the bands is an explanation of how the bands were produced and what they might indicate.

Interpretation of Gel Banding Patterns

Three patterns (see Figure 67) are generally visible in side-by-side PCR product profiles where “screening oligos” were used as primers compared to the PCR products that represent the entire length of the inserted DNA (produced by the “sequencing oligos”). For each pattern comparison the length of the entire inserted DNA (produced by PCR using “sequencing primers” alone) is on the left side.

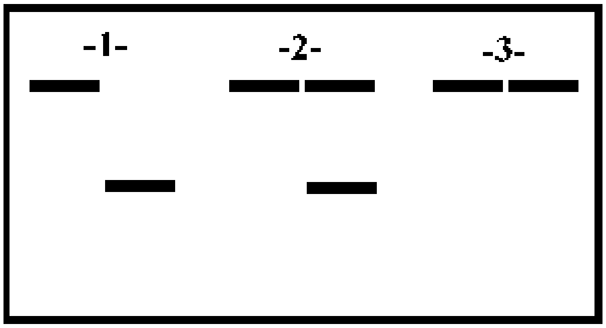

Figure 67. PCR product patterns obtained during secondary screening.

1. Only a shorter length fragment is produced in comparison to the entire inserted DNA fragment length.
2. Both a shorter fragment and as well as a fragment the same length as the original inserted DNA are visible.
3. Only a fragment the same length as the original total DNA insert is produced and no shorter fragment is visible.

In the first case (1) it appears that the “screening oligos” (which are simply synthesized sequences of repeated DNA) compete strongly with the sequencing primers so that the primary product is a shorter fragment that indicates the distance that the microsatellite repeat unit that matches one of the “screening oligos” is away from one edge of the inserted DNA sequence. Competition for primer binding appears less pronounced when both original length and shorter fragments are produced (2). However, in the last example (3) the “screening oligos” do not appear to bind at all, so no shorter fragment is produced. This last case would indicate that no microsatellite repeat region matching the screening oligos exists.

Figure 68 shows diagrammatically how the various primers (that initiate the DNA replication during PCR) might bind to the original template DNA and produce the products that are visible as stained bands on gels:

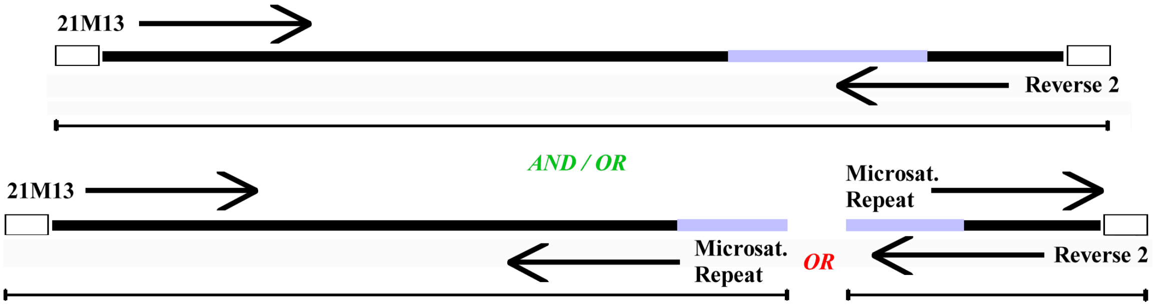

Figure 68. Potential primer binding sites during secondary screening PCR. Only "sequencing primers" (top). "Sequencing primers" along with "screening primers" (bottom). Shaded portion of fragment represents region of DNA inserted into the pUC19 plasmid containing microsatellite repeat units. Labels and arrows identify potential primer binding sites.

Locations of primer binding sites are labeled and length of fragment produced is represented by the bar beneath. The shaded area represents a region of DNA containing microsatellite repeat units.

"Sequencing primers" bind at the ends producing a fragment that represents the entire length of the DNA that was inserted into the circular plasmid and transformed into *E. coli* for plasmid copy multiplication. However, if another primer binds at a point between the "sequencing primers" there is potential for production of a shorter fragment as well. These possible alternate (or additional) short products are viewed as fragments lower on a gel and have a microsatellite repeat as one of their end points.

In order for the PCR amplification reaction to occur one primer must bind to a single strand of DNA and begin replication in one direction as another primer binds to the complementary (matching) single strand of DNA and initiates replication in the opposite direction. The products produced by the single primers moving along the complementary strands of DNA in opposite directions must overlap in order for high-copy replication to occur (see Appendix C, page 172, for more details on PCR).

If one of the “screening oligos” is complementary to a sequence (identified by the shaded region in Figure 68) in the larger fragment a shorter fragment may be produced, or possibly be produced as the primary product. Figure 69 represents possible simplified combinations of products produced. Usually only one of the shorter fragments would be produced because the “screening oligos” that are added are complementary to only one strand of the DNA. But inversions of repeat sequences or mixes of different types of repeat units can lead to possible multiple short sequences.

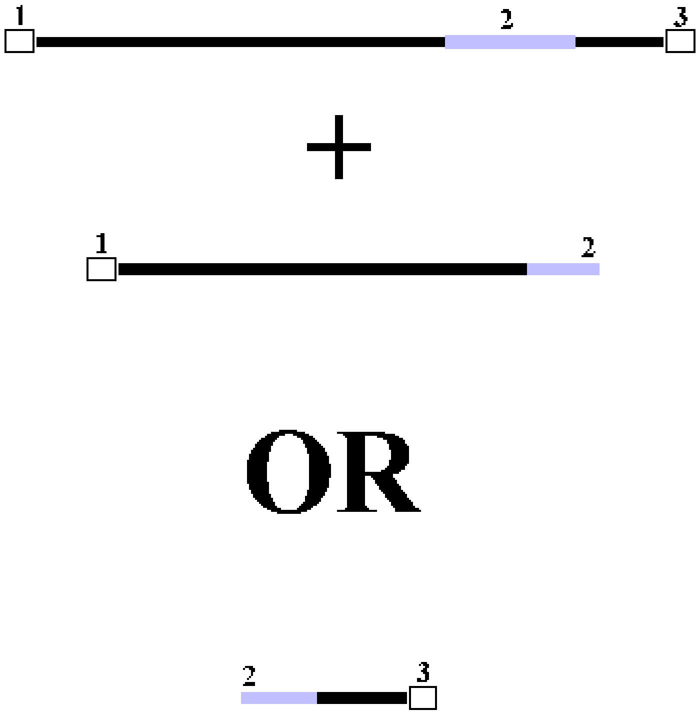

Figure 69. Examples of possible PCR products produced when “screening oligos” are used with “sequencing oligos” and a microsatellite repeat region is present within the inserted DNA.

The arrangement of the fragments visible in Figure 69 also represents the order they would appear in on a gel (longer fragments at the top and shorter at the bottom). The photograph of a gel in Figure 70 provides examples of the previous descriptions. It is provided as a “real-world” example of how products look on gels (as opposed to a stylized diagram with perfect products for unquestionable identification). The only alteration from the picture straight from the gel documentation system (Alpha Innotech IS-1000 Digital Imaging System) is inversion of color for easier band identification.

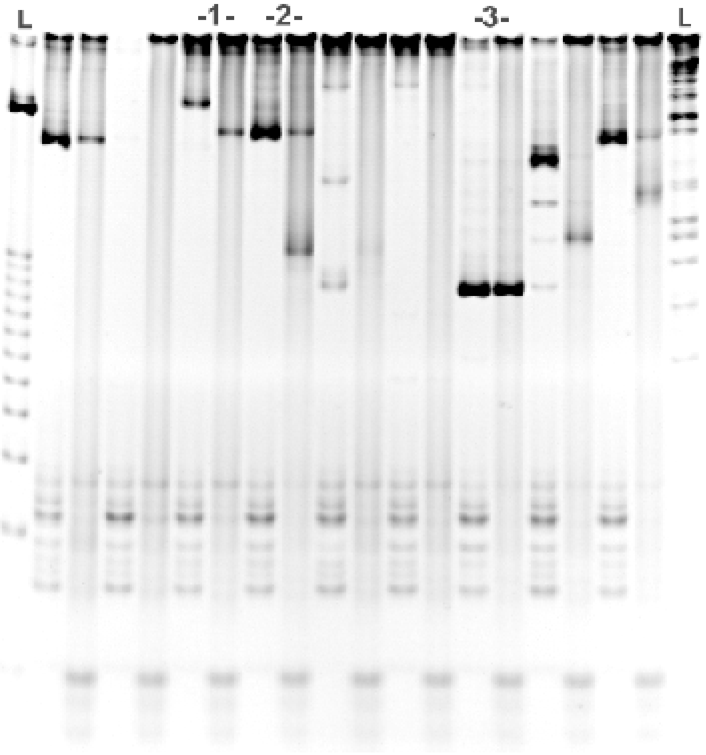

Figure 70. Example of a gel documenting the various banding patterns identified in Figure 67. Numbers above paired lanes match product types identified in Figure 67.

The numbers above the paired lanes in the gel (Figure 70) match the types of products identified in Figure 67. “L” indicates the molecular weight ladders used to determine fragment lengths. Short bands near the bottom are produced by primers interacting among themselves during PCR.

APPENDIX C – PRINCIPLES OF PCR AND ELECTROPHORESIS

Principles of PCR

PCR (polymerase chain reaction) is a process used to make many copies of select regions of DNA. The process involves the three repeated steps shown in Figure 71.

1. Template (original) DNA is first denatured or broken apart. The double stranded helix is separated so that single stranded DNA exists. This is accomplished by heating the DNA to 94°C.
2. Temperature is then reduced in order to allow the primer to bind to the single stranded template (original) DNA.
3. Temperature is raised to the point where optimum extension (replication) of the DNA takes place.

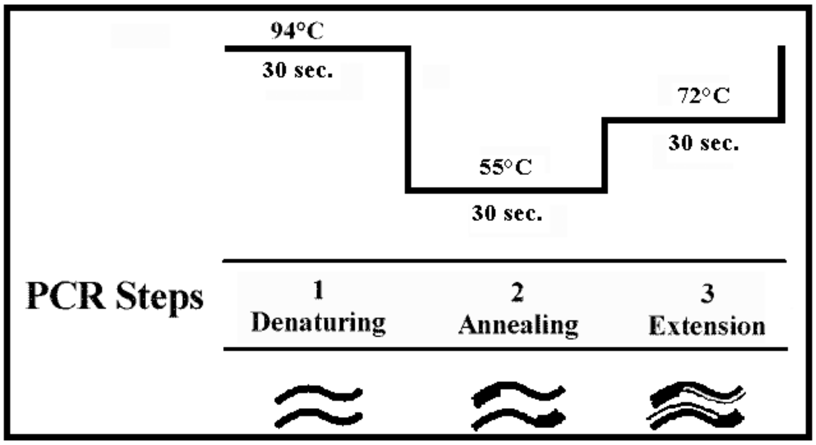

Figure 71. Overview of polymerase chain reaction (PCR) cycles.

In order for the process to take place a mix of the original/template DNA, primers, individual nucleotides (dNTP’s - A [adenine], T [thymine], G [guanine], C [cytosine]), $MgCl_2$, a buffer solution, and *Taq* DNA polymerase (the enzyme responsible for replicating the DNA, originally extracted from bacteria discovered in hot springs -

Thermus aquaticus) must be made up. Primers are simply small segments of DNA that are synthesized to be complimentary to a specific region (or regions) of template DNA. Figure 72 is an example of a primer. It is characterized by having A's matched with T's (or T's with A's) and G's matched with C's (or C's matched with G's).

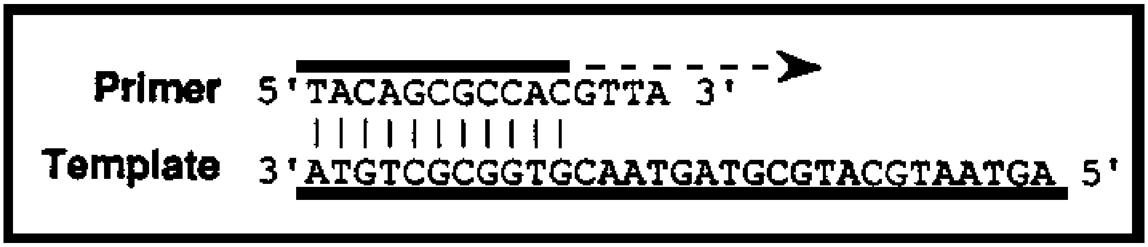

Figure 72. Example of a primer used in PCR.

A diagram of the process of replication is shown in Figure 73. The following is an explanation of what is depicted:

1. Isolated (extracted) double stranded DNA is shown.
2. Separated single strands of the original DNA template are shown with the primers (looking like small bugs) bound to them.
3. Extending from the point where the primers were first bound the *Taq* DNA polymerase replicates the DNA strands. (The wavy lines indicate that DNA extension has progressed past the viewable region. Notice - that the DNA is replicated beyond the area that is desired to be copied [which is the region depicted as a straight line.])
4. Once again the double stranded DNA is denatured to provide single stranded DNA for the primers to bind to.

5. Replication of the DNA again takes place. (Notice - that half of the copies of newly replicated DNA are now limited in their extension to the point where a primer can bind.)
6. Again the double stranded DNA is denatured and the primers bind (anneal).
7. DNA replication takes place once again. (Notice - that the more the replication process is performed the higher the percentage of DNA fragments that are limited in their length to the distance between where the two primers bind will be. This region is depicted as a straight line.)
8. Typically the number of times the PCR process is repeated is between 25-35. A machine called a thermocycler is normally used to automate the process. This machine also allows the time spent at each temperature and the temperatures themselves to be modified in order to optimize the process.

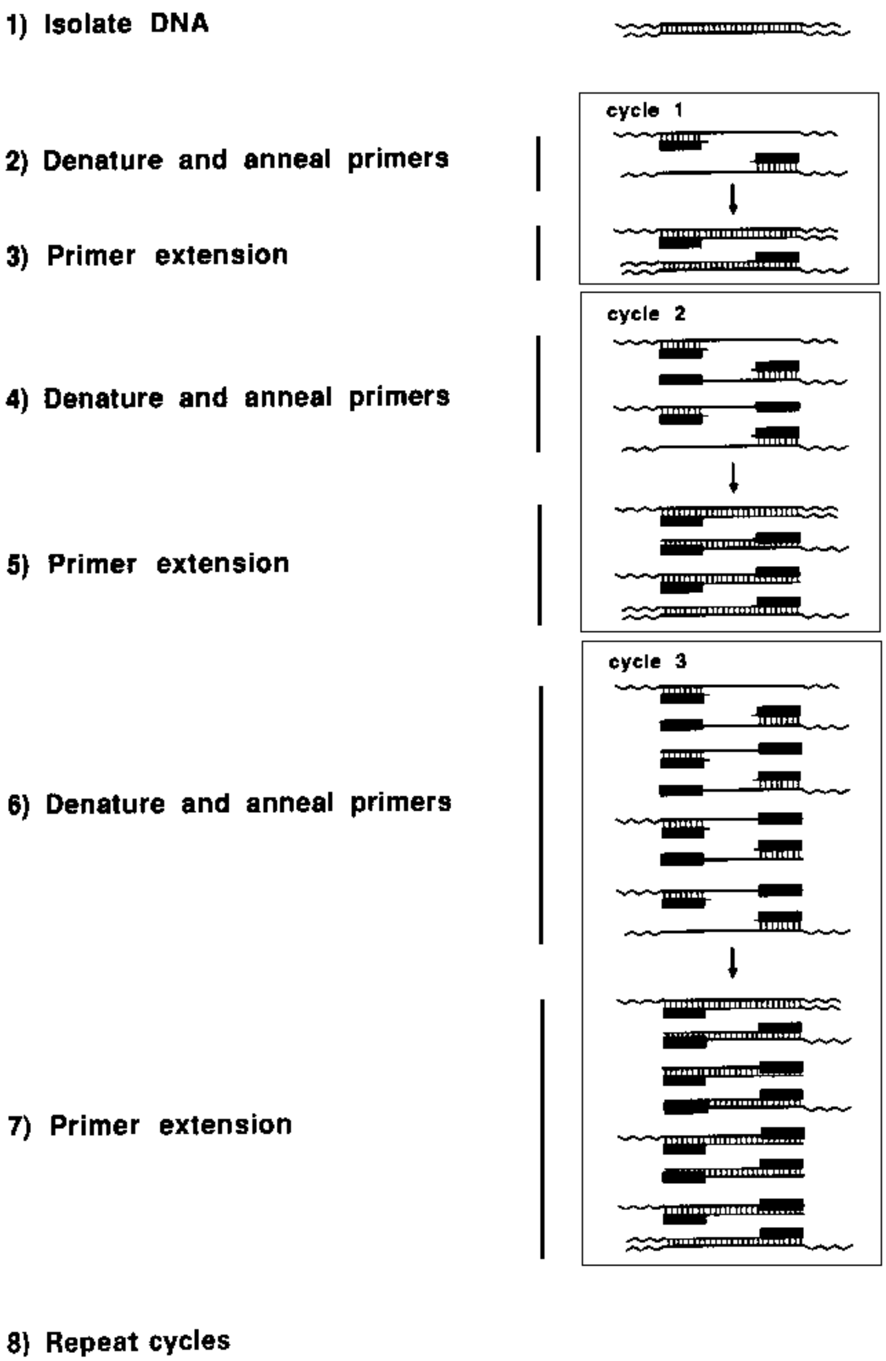

Figure 73. Summary of polymerase chain reaction (PCR) for amplification of specific DNA fragments.

PCR is used for many genetic analyses ranging from paternity testing and criminal forensic analyses to speciation and phylogenetic tests to examinations of variation between species' subpopulations. Depending upon the specificity of the primers that are used the number of locations within the genome that are copied and amplified varies. This means that for very specific primers either one or two sizes of DNA fragments will be produced while for other less specific primers many different sizes of DNA fragments are produced. The various sizes of DNA originate from multiple sites within the genome where the primers can bind and *Taq* DNA polymerase can replicate the fragments.

Factors Affecting PCR

Since the polymerase chain reaction (PCR) is simply a set of chemical reactions taking place, changes in any of the components of the recipe followed can affect results. Below is a list of recipe components and their activity within the reaction as well as procedural steps that affect outcome. The desired outcome of reactions is to obtain clearly identifiable bands that are reproducible and consistent with the product desired from the primers used.

- Working on ice – in order to keep all components cool while combination is taking place is valuable. It ensures that reactions do not begin before everything is mixed together and thermocycling (going through the individual steps at set temperatures and times) begins.
- PCR grade water – ultra pure water with no contaminants that might interact with or affect the overall reaction.
- $MgCl_2$ – acts as an enzymatic cofactor in the reaction. A general starting point for concentration is often 2 mM in the final reaction. Changes in $MgCl_2$

concentration affect PCR results greatly. For this reason altering $MgCl_2$ concentration (up and down) is a major part of most optimization procedures.

- 10x Buffer – maintains a more stable pH during reactions, ensuring that an optimal value is maintained for reactivity. Usually the buffer delivered with the *Taq* polymerase is used with no modification. However, changes in pH can significantly affect the PCR reaction and improve or reduce results.
- dNTP's – are the source used to construct additional copies of the DNA. They are individual molecules (A [adenine], T [thymine], G [guanine], C [cytosine] – also referred to as "bases") put together to form DNA. Generally a set amount of these "building blocks" is added to each reaction. However, changing overall dNTP concentration by adding more or less of one or more bases can affect results.
- Primers – match specific sites on the original template DNA. A's match with T's in the template (and the T's with A's) and G's match with C's (as well as C's with G's). At the point where primers bind to template DNA duplication of the remaining single DNA strand (not matched up with the primer) can take place. Replication moves along the template DNA in the 3' to 5' direction (see Figure 72 on page 173). Most of the time a standard quantity of primers is added (which is often much more than will ever be used), however, adding more or less can affect overall reaction results.
- *Taq* polymerase – is the enzyme responsible for replication of template DNA by addition of bases (A, T, G, or C) beginning at the primer binding site. Adding more or less *Taq* can significantly affect results. However, most of the time a standard amount is used and other components within the reaction are altered.

- Template DNA – is the original DNA that is to be duplicated. Adding more or less template DNA may affect reactions significantly. However, concentration (above some adequate value) usually doesn’t hurt reactions. Having low quantities of template DNA can lead to few copies being created and poor results that are hard to visualize electrophoretically on a gel.
- Mineral oil – is added to prevent evaporation of the reaction mixture during the heating and cooling cycles of PCR. Without addition of oil and tight covering of reaction tubes complete evaporation will occur before the end of the reaction. Such a change in volume will affect concentrations of all reactants.
- 94°C – denaturing temperature is variable as well. Temperatures used range from 91-94°C. Extended periods at high temperatures inactivate the *Taq* and negatively affect its enzymatic activity.
- 72°C – extension temperature is set at the point where *Taq* polymerase is most active. It is not generally changed.
- Annealing temperature used is highly variable and dependent upon primers included in the reaction. Optimum annealing temperature calculations can be made based upon primer sequence, but experimentation with values above and below the calculated optimum is often very valuable.

Principles of Electrophoresis

Figure 74 describes some of the principles involved in electrophoresis. Gels are typically made of agarose or polyacrylamide. Altering the concentration of agarose or polyacrylamide affects how fast DNA can move through the gel. During “gel runs” a buffer solution provides contact between the positive and negative electrodes and the gel. Since buffer electrolyte concentrations affect flow of current it is important that the surrounding buffer be the same as the liquid used to hydrate the gel itself. DNA fragments are negatively charged. When a current is passed through a gel DNA is attracted toward the positive electrode. Smaller pieces of DNA move faster (and thus are found farther down the gel) because they can move through small spaces within the gel matrix easier. By using a "marker" with DNA fragments of known length estimates of DNA fragment size in samples can be calculated.

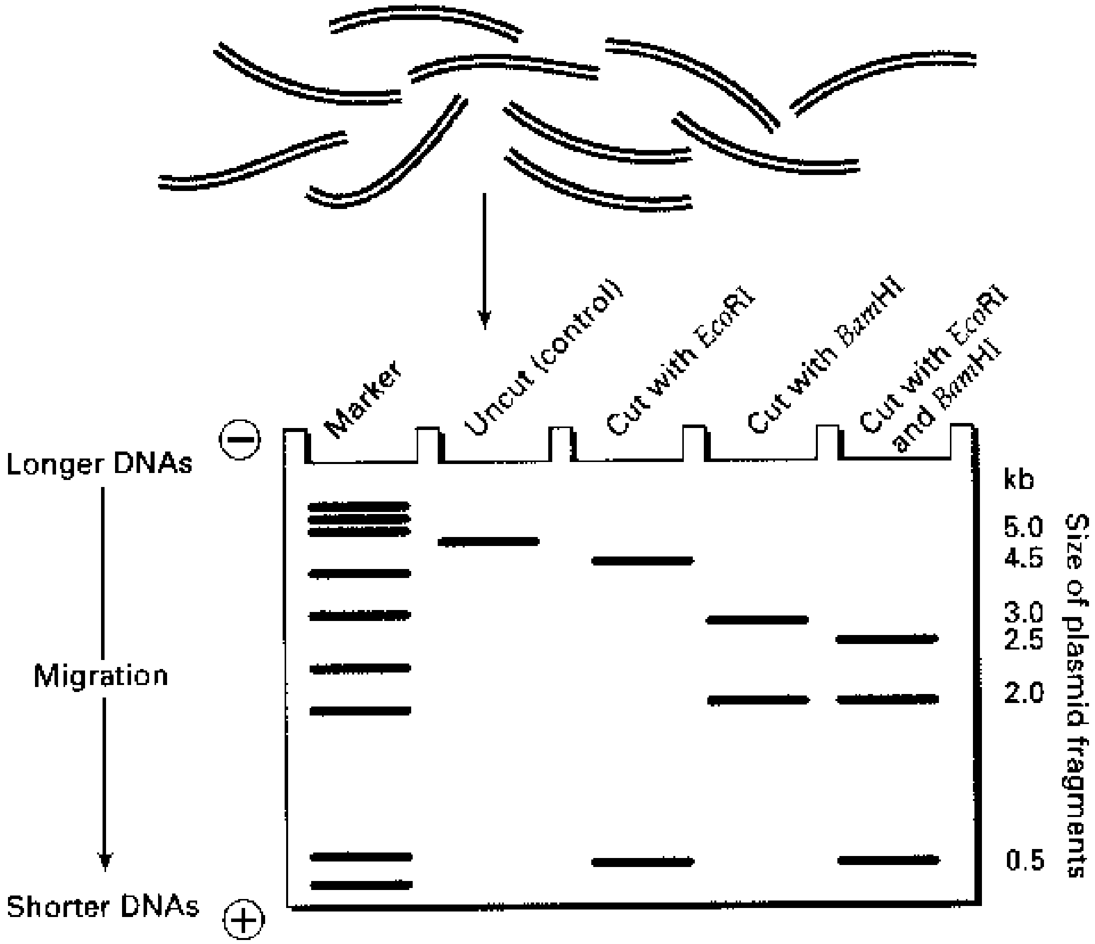

Figure 74. Overview of electrophoresis.

APPENDIX D – PREPARATION AND USE OF SPREADEX® POLYMER NAB GELS FOR SEPARATION OF MICROSATELLITE ALLELES

Prepared high resolution Spreadex® gels have been available for several years and been described for use in single strand conformational polymorphism (SSCP), three base pair gene deletion (Elchrom web site: www.elchrom.com), as well as microsatellite repeat polymorphism (Luqmani *et al.* 1999) detection with Elchrom Scientific's own gel electrophoresis apparatus. However, we successfully used our own prepared gels made with Spreadex® Polymer NAB and a common vertical gel rig for high resolution screening of microsatellite repeat polymorphism with excellent results.

Electrophoresis equipment consisted of an SE 600 Series vertical slab gel unit (Hoefer Scientific Instruments) with 16 cm long glass plates. Up to two gels (approximately 50 samples) may be run at once. The apparatus permits total submersion of gels in running buffer, which ensures even dispersal of heat generated. Without dispersal of heat gels warm unevenly during runs and produce a characteristic "smile" effect, since DNA moves faster through the warmer central region. Both 1 mm and 0.5 mm thick spacers have been used in gel preparation and provide satisfactory results. (0.5 mm spacers and combs were hand-made from thin plastic sheeting. Cost savings associated with reduced materials is the primary reason thinner gels are used, while still maintaining high quality visual results.)

Product literature states that Spreadex® Polymer NAB (Native Acrylamide-Bis) has been optimized for use with gels made with a ratio of acrylamide to N,N'-methylene-bis-acrylamide of 29:1 and with 1x TAE running buffer. In short (8-10 cm) precast Spreadex® gels products must be run nearly to the end of the gel for satisfactory resolution. The short distance products travel helps to ensure little diffusion and band blurring occurs. However, we have found that a longer gel (16 cm) allows for maintenance of a safe buffer zone around the fragment sizes of interest, making determination of approximate electrophoresis run time necessary for good separation without losing product easier. An additional benefit of this greater area in front of and behind the fragment size of interest is the greater likelihood that very different allele sizes are still visible on the gel rather than possibly being eliminated, having run completely off the gel.

Percentage of acrylamide used in Spreadex® gels must be optimized for best separation of DNA fragments within a specific size range. A table of gel concentration recipes suggested for optimal separation for a range of DNA fragment sizes is provided in the product literature and on the company's Internet site (www.elchrom.com). These ranges and recipes are for 8-10 cm gels and may be adjusted to produce optimal results for longer gels. Running time required for optimal separation of DNA fragments within a size range varies with gel concentration and must be determined experimentally. A typical run time to separate 75-100 bp fragments with 1-2 base pair length differences clearly visible on a 16 cm long 5% Spreadex® gel is 75 minutes at 300 volts. (See section in Chapter 2, on page 26, concerning gel production for a specific example.)

Because of the high separation ability of Spreadex® gels, samples must enter the gel evenly. The recommended means of achieving this is to pour the Spreadex® gel and allow a 0.5-1 cm space near the bottom of the comb to remain. A layer of 50% methanol is then poured over the gel layer while the Spreadex® "running" layer solidifies. After a short period a visible interface between the gel and methanol develops and 4% acrylamide solution (without Spreadex®) is used to rinse the upper portion of the glass plates following removal of the methanol. TEMED is then added to the remaining 4% acrylamide solution and used to pour an upper gel layer that the samples run through before reaching the lower Spreadex® containing running gel. Consequences of imperfections in well shape (reflected in ultimate band location and appearance) are substantially reduced when a top 4% acrylamide layer is used.

Gels were stained with ethidium bromide for 25 minutes followed by 15 minutes of de-staining. An IS-1000 Digital Imaging System (Alpha Innotech Corporation) was used for gel documentation and analysis.

APPENDIX E – MICROSATELLITE PRIMER SETS TESTED

The following are microsatellite primer sets discovered (along with a few others obtained elsewhere) and tested to evaluate possible usefulness in paternity analyses. Not all primer sets discovered and tested were identified as potentially useful for paternity testing. After preliminary testing some contained no individual allelic variation or no recognizable product (see Results and Interpretation for detailed information, beginning on page 130). Microsatellite sequences discovered were submitted to GenBank and may be found there by using the search term "Sonnentag" on the following web page: http://www.ncbi.nlm.nih.gov/

Note that the GenBank submission names differ from laboratory notebook identification by the addition of a prefix ("AC(caam)"). When available all sequence information that follows is provided in its official GenBank format. Additional primer sets tested (with sequence information if available) are listed after microsatellite sequences submitted to GenBank.

Microsatellite ID	Page
Discovered:	
14 = 14(2) in lab notes	185
22	185
29	186
30	187
40	187
42	188
46	189
50	189
56	190
60	190
66	191
119/147	192
196	193
Additionally Tested:	
Am(GT)-2	194
Am(GT)-4	194
Am(GT)-7	194
Co-18A	194
Co-18B	194
Co10F11	194
Co12E10	195
EST2	196
EST4	196
CCF-2/102	197
CCF-4/70	197
CCF-6/37	197
L780	197

Discovered Microsatellites:

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DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)14 sequence.
ACCESSION   AF452618
VERSION     AF452618.1  GI:17981045
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 317)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 317)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
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       61 acttctaggg gaatttctta gaaaaaggaa cctcaggccc aagagctata cttttccaac
      121 caggaagtca cttccagttt cagaaaaaga tcccagccca gatcagctta aaacaacctg
      181 agcaccctgt aaacctggag aaatgatctc ccatgggcct tagaaagtat gtgtgtgtgt
      241 gtgtgtgtgt gaggggtctg taatgtcttc acgggccaca ccttgaagaa atacggagac
      301 tgaaaattct gatacag
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LOCUS       AY035298                 335 bp    DNA     linear   VRT 26-JUN-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)22 sequence.
ACCESSION   AY035298
VERSION     AY035298.1  GI:14571507
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 335)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
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REFERENCE   2  (bases 1 to 335)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
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                     /sex="male"
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                     /country="Ecuador: islet Caamano"
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                     /rpt_type=tandem
                     /rpt_unit=ac
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       61 gttataaata aatacactga attgttccca taaatagatg accaaaatgg gttgtttgac
      121 cttttaaaga cctatacaca gtctcaccca aaactgtttg aaggaacacc ttcttcgaca
      181 aaacacacac acacacacac acacgtccta cattgcatca tgactgaatt agaccttttt
      241 tctgtaagtg ggtggcctta ataatgagag ctacgttggt ggtggtgccc acacacagag
      301 ctttggctgg tacctttgcc tacagccttt caagt
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LOCUS       AF297084                 483 bp    DNA     linear   VRT 02-OCT-2000
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)29 sequence.
ACCESSION   AF297084
VERSION     AF297084.1  GI:10444403
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 483)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 483)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-AUG-2000) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
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                     fragment length"
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        1 gatcagacaa aatccactga aagagctggg acaaactaca gtttatcttg tctatacttg
       61 ctctatcaat ctatattcga aagtagttct tccacgatat ggttggggac tctgcagccc
      121 actaggtgtt gctgaactgc aactcccagc attcctaatg attggctgtg ctggctaaga
      181 ctattgggag ttgcagtcca ataacatctg tccctaccct tgctctaagg tcagaaagaa
      241 ctgacttccc atgactcact gaagggcctt ttcacacttt acaatgctct gtgtgtgtgt
      301 gtgtgtgtgt gtgtgtgtct gtctgtctgt ctgtctgtcc aaaagtatgc tacttgccaa
      361 aatactggga ctgattgcaa ttacatgaga agcatatgtt cagcattaag tgtgaattta
      421 ctgctgcatg tataaaactg ctttaaaaag aaaacactgc aaagtgatgg gacaagtttg
      481 atc
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LOCUS       AY035299                 386 bp    DNA     linear   VRT 26-JUN-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)30 sequence.
ACCESSION   AY035299
VERSION     AY035299.1  GI:14571508
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 386)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 386)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
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                     /rpt_unit=tg
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      121 gcatgtctaa tttccactgt gcaaaaagtt tcagaatttg gaatgttcat aagtgtgata
      181 ttcagtccta cactgcacaa atttgtatgt ttgtttgtgt gtgtgtgtgt gtgtgtgtgg
      241 atttaggcca atgagctgaa aatagctttc aaagttctag atattttata accctgttga
      301 aattttccct aaggcacttg taggtattag gaataagagt gaataatttt tttccctatt
      361 tctatcataa gcattattaa aatgat
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ACCESSION   AY035300
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VERSION     AY035300.1  GI:14571509
KEYWORDS    .
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            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 277)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 277)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
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      121 tttcacctct tttccttctc aataggctgt gtgtgtgtgt gtgtgtgtgt gagtgcattg
      181 gtgcatgaga atattttcat tcaataaaat tcatttattt gatatgtttg tctggctttt
      241 tggcttggcc tgaccccgta aagttgagta aaagatc
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VERSION     AY035301.1  GI:14571510
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
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            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 251)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 251)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
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      121 aggtatgact ggtagcaact tcaactctta aaattcattt cacatctggt tccaaacaca
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LOCUS       AY035302                 362 bp    DNA     linear   VRT 26-JUN-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)46 sequence.
ACCESSION   AY035302
VERSION     AY035302.1  GI:14571511
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
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            Amblyrhynchus.
REFERENCE   1  (bases 1 to 362)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 362)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
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                     /country="Ecuador: islet Caamano"
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                     /rpt_type=tandem
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     repeat_region   323..340
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ORIGIN
        1 gatctacttg tgaaactatt ttaacaccag cacagtcttc caaccacaca atcattggct
       61 ttgtaaacta acacagtttc aaataattaa agcacacaca tatggaatat cccattacta
      121 gatgttctac tgtctaagtt ttcatcacac acacacacac tcacacacac gcacatacac
      181 aggcatcaga actctttctc caatggtgta ggctcactac actaaattta tgagtattac
      241 aatcccagga ccgagcaaag acattttgct gcctgagaca aatgataaaa tgggaatggg
      301 aatgtgtgtg catgtgcgcg cgcacacaca cacacacaca tattctactt taatgataga
      361 tc
//

LOCUS       AY035303                 312 bp    DNA     linear   VRT 26-JUN-2001
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```
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)50 sequence.
ACCESSION   AY035303
VERSION     AY035303.1  GI:14571512
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 312)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 312)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
     source          1..312
                     /organism="Amblyrhynchus cristatus"
                     /db_xref="taxon:51208"
                     /sex="male"
                     /tissue_type="blood"
                     /country="Ecuador: islet Caamano"
     repeat_region   174..211
                     /note="microsatellite AC(caam)50"
                     /rpt_type=tandem
                     /rpt_unit=ct;gt
BASE COUNT       74 a     45 c     77 g    116 t
ORIGIN
        1 gatctggtgt ttttagggtg tttatgccat aatgtgttta ccattatgtt gtacagaggt
       61 ttaacacttc ctgattataa aaaagaaatt ggcatagctg agaaacatta atttttgtag
      121 tcctccaaag taacatttct aagctcttta gaaagtgact gctgtgtagt caactctctc
      181 tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tagttatggt ccttggcttg ggaaagtgat
      241 taaccagctt ctaattgaga gggagttgtg tgttctcgat gcattcttcg ttcagagatg
      301 ctcagcatga tc
//
```

56 (not submitted to GenBank due to very short repeat region and no variation, so questionable identification as a microsatellite)
Primers indicated between ' marks:

```
5’-GATCCACTGCTCACTGTCATGGAGGCCCCATGCTGCCACTGCAACTAAGCAGAGTT
'TTTTTGCTGTGTCTGGATTTTG'ACAGACTGTGGTTAGTCTTAACTATGGTTTAGGAA
AACAAACCAATTTGAAATTATGACTTATGATTCTAGATGTTTCTCTGAAAATATTTTTA
AGATTATCTATGATTCCATATTTAGACATGTGAGTAAAACCAGGTTAATTGAAAACAGA
AACAAACATTTCTATTTCATTATGAGTTCTCCACTGTATGTGTGTGTGCATGTGCATGC
ATGCAGATAAGTCCAGGACCTATG'GACATAGCACTTACCAAACCAT'AGTTTAGCATT
CCATTCCAATTAGATC-3’
```

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LOCUS       AY035304                 282 bp    DNA     linear   VRT 26-JUN-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)60 sequence.
ACCESSION   AY035304
VERSION     AY035304.1  GI:14571513
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
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            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 282)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 282)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M. and Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-MAY-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
     source          1..282
                     /organism="Amblyrhynchus cristatus"
                     /db_xref="taxon:51208"
                     /sex="male"
                     /tissue_type="blood"
                     /country="Ecuador: islet Caamano"
     repeat_region   96..139
                     /note="microsatellite AC(caam)60"
                     /rpt_type=tandem
                     /rpt_unit=tc
BASE COUNT       65 a     85 c     35 g     97 t
ORIGIN
        1 gatcccaaat atcaaaaatg tgctgttttg ataagacttg caatcatttt taaacattca
       61 catattaatc ttgagtcagt cagtcagtcc gtcagtctct ctctctctct ctctctctct
      121 ctctctctct ctctctctct ttctgtctcc ttccttcctt cctttcctcc ctctccccac
      181 cccaatccag aataatctga atgtggaaat aattactcct aaaggcagct gtccaactgg
      241 gaacacttcc tgcattcatg gaagtggatt ctacacgaga tc
//

LOCUS       AF452619                 449 bp    DNA     linear   VRT 27-DEC-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)66 sequence.
ACCESSION   AF452619
VERSION     AF452619.1  GI:17981046
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 449)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 449)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
     source          1..449
                     /organism="Amblyrhynchus cristatus"
                     /db_xref="taxon:51208"
                     /sex="male"
                     /tissue_type="blood"
                     /country="Ecuador: Islet Caamano"
     repeat_region   259..274
                     /note="microsatellite AC(caam)66"
                     /rpt_type=tandem
                     /rpt_unit=t
     repeat_region   276..303
                     /note="microsatellite AC(caam)66"
                     /rpt_type=tandem
                     /rpt_unit=gt
BASE COUNT      116 a     79 c     96 g    158 t
ORIGIN
        1 gatccccctc tttttgtttc tgacaaccag tcagtattgc tgcttttgct ttccaacaca
       61 tcatgacata gttattcctg tataagaaat acggggagtt gaataccttа tttggggata
      121 attggaagag cagtcctata gatatagctg ttaaatttct acaccactct aataaattgg
      181 taacagaatg ctccaaaccc tccatttgta ttgagtgctt cttttaagca cacttcttgg
      241 attgcaaata cccaagaatt tttttttttt ttttggtgtg tgtgtgtctg tgtgtgtgtg
      301 tctgcatgga catcagacac tgaaagaggg gagagggaag gtccctattt atcatgcacc
      361 ttgcaatcgt ggctgaatga tgtatagtga ttacattttt tttctaattg gcaggcttcg
      421 gaaactgcta tgggaattgg tgacaagat
//

LOCUS       AF452620                 556 bp    DNA     linear   VRT 27-DEC-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)119/147 sequence.
ACCESSION   AF452620
VERSION     AF452620.1  GI:17981047
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 556)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 556)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
     source          1..556
                     /organism="Amblyrhynchus cristatus"
                     /db_xref="taxon:51208"
                     /sex="male"
                     /tissue_type="blood"
                     /country="Ecuador: Islet Caamano"
     repeat_region   434..455
                     /note="microsatellite AC(caam)119/147"
                     /rpt_type=tandem
                     /rpt_unit=tg
BASE COUNT      143 a     91 c    119 g    203 t
ORIGIN
        1 gtttataaga cagagctttc tctgtagtag catcttgtat gcagaactgt ctcccaaggg
       61 aacacagacc tgatacaccc cctgttcaac tactgctgga ctgccaagac tatatatttc
      121 caagcactat gaccatctct gtgctatgac catttctgca tgacactgtt atagtgctat
      181 tattccattt ttaacattct gtggaatttg gaggtttgta gtctactgag gccgcagacc
      241 tctttagctg agaattctaa gtgttcctcc ctaaactgca aatgccagga tatcattaga
      301 tgttgaaagt ggaataatag cactataaca gtgcaatgtg gaaatggctt gtgattaata
      361 ctggttttca gtattgtttt tattcttgaa gaatgctgga atctttgaac tggttttgga
      421 aagctgtttc gtttgtgtgt gtgtgtgtgt gtgtgagtat ttgttttaaa actgtttctg
      481 agtaatgttt ttgaaaaatg tgggcttctt agaattgacc tttagaagtt gttttttaaa
      541 atctgttttg gacaga
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LOCUS       AF452621                 503 bp    DNA     linear   VRT 27-DEC-2001
DEFINITION  Amblyrhynchus cristatus microsatellite AC(caam)196 sequence.
ACCESSION   AF452621
VERSION     AF452621.1  GI:17981048
KEYWORDS    .
SOURCE      Amblyrhynchus cristatus.
  ORGANISM  Amblyrhynchus cristatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Iguanidae; Iguaninae;
            Amblyrhynchus.
REFERENCE   1  (bases 1 to 503)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Discovery and characterization of microsatellite markers for
            paternity and population analyses in the Galapagos marine iguana,
            Amblyrhynchus cristatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 503)
  AUTHORS   Sonnentag,J.A., Carter,R.L., Sands,J.F., Wikelski,M.C. and
            Hayes,W.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2001) Natural Sciences Department, Loma Linda
            University, Griggs Hall, Loma Linda, CA 92350, USA
FEATURES             Location/Qualifiers
     source          1..503
                     /organism="Amblyrhynchus cristatus"
                     /db_xref="taxon:51208"
                     /sex="male"
                     /tissue_type="blood"
                     /country="Ecuador: Islet Caamano"
     repeat_region   268..281
                     /note="microsatellite AC(caam)196"
                     /rpt_type=tandem
                     /rpt_unit=at
     repeat_region   282..307
                     /note="microsatellite AC(caam)196"
                     /rpt_type=tandem
                     /rpt_unit=gt
BASE COUNT      170 a     74 c     96 g    163 t
ORIGIN
        1 gatctttttc tctcattcag tatcaaaggt gtgttagaac tattacaggc tagcacatgc
       61 atgcttttca aggagagagt ggaatacttc ctgataaata aggaaaaggc gaacttttcc
      121 aaggctcttg ccaaacatag atttccaatt tagctagggt ttgttccact gtgccaaatg
      181 aagatgagca gaatcagtct tgtgttagga tagatagaga gaatctaata gcaaaatatt
      241 tggtatattc tacggtagca ttttgcaata tatatatata tgtgtgtgtg tgtgtgtgtg
      301 tgtgtgtcca tactttttta aagaactaag gtgatataga aattcttcaa gatgaaaatg
      361 aaacagcaca acaacaaaaa caacaaactt catgccatag attattgagt agcttcagtg
      421 ctttgaatca tggcatggca tgattcattt cattcttcaa taactaaata aataaattaa
      481 atagtatcaa atgttttgcg atc
//
```

Additionally Tested

Sequences received from Kornelia Rassmann (08/1997). Primers indicated between ' marks:

Am(GT)-2 (allele no. 14)
Note: Primers designed and used in analysis (2nd Am(GT)-2) indicated by | marks.
5'-CCCTTCAGTAAACTACAAACCCCAGAATTCCACAGGATGTTGAAAGTGGAATAATG
GCACTGTAACAGTTTAGTGCAGAAATGGCTACTCAGAACAGTGTAAGCCTGAATATATC
AAGCAGCATTTTCTGGTTATTTTCAGTTAC|CAAAGGGCATTATCA'TC|GGCATTTTA
AATAGA'ATTTTTTTTGCATGTTTTAGGTTTTTCCCGCCCCCCAAAAAAGTGACTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTTAATGGTAATGAATT|ACTTTGGTGGTTCTGTT
|ACATATT'TCTTAATGAATTATTTT'CCAAAAGTAG-3'

Am(GT)-4 (allele no. 7)
5'-CCCTTTCAAGACAAATCTT'TTATGGATGAGCAATAC'AACTACTATCCCAAATCA
CACATACTTCCCCTTTTTAACATGTGTGTGTGTGTGTGTGTATGTTAGACCAGACTGGT
CCCCTCTTGTGGATGAAACAAACATAAAGGTCTTTGGAAGGGAGATGCACACACAAGAG
AGAAGCCTCGGTCACTTCTCTTCTCTTCTTCCGTCCCAACC'CTACAAGGCATATATAC
'ACTTATCCACAATTCAGTATTACAATACAGGCAGTATCTCCATTTCACATATAGATGC
ACTACAAACTAGAG-3'

Am(GT)-7 (allele no. 11)
5'-CCCTCGAGCACACATTATACTAATCACAAGGATCTATGCCTCTGTGG'TAAGTTTA
TCTCCTGCC'TTCTATTCATATGCTAAGGCACAACTTACTACATTAATCCTAGTCATCA
CTACCAGACACACACACACACACACACACACACACATTCACAC'TCACATTGCTGGTAA
CA'GCAGACCTCTAACCGAATTGGATCCAGGAAATCCTACTGTAAGGCTGAGCAACATT
TGGGGGACACATACCTGCCCCATGCCTGCTGCCACATCTGTTGGACAAGCATTTGCAAG
ACTCTCCAAAA-3'

Co-18A (land iguana)
5'-'ATAATGCTCTGAAATGGTGTG'AGAAATGAATTTCCCTAAATGAATATTTGRACT
GAGTAATANTTTATTGCAAGATCTGCAAATGAGGAACTTAACAGGCT-(CTAT)$_n$-(CCA
T)$_n$-CCACATTATTAATAGGTTCAA'ATTATGGATGTCAGAATGAGT'-3'

Co-18B (land iguana)
5'-'GCATGGAAAACTTGATTATAT'GCTAAGAATTCC-(CTT)$_n$-(CCA)$_2$-CCT'TAT
GTCAAAGATAACAGGGAA'-3'

(marine iguana)
5'-'GCATGGAAAACTTGATTATAT'GTTAAGAATT-(TCC)$_n$-A-(CCA)$_5$-
CTC'TATGTCAAAGATAACAGGGAA'-3'

Sequences selected from related species for testing:

```
LOCUS       AF159512                 376 bp    DNA     linear   VRT 28-FEB-2001
DEFINITION  Ctenophorus ornatus microsatellite Co10F11 sequence.
ACCESSION   AF159512
VERSION     AF159512.1  GI:5639933
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KEYWORDS    .
SOURCE      Ctenophorus ornatus.
  ORGANISM  Ctenophorus ornatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Acrodonta; Agamidae;
            Amphibolurinae; Ctenophorus.
REFERENCE   1  (bases 1 to 376)
  AUTHORS   Lebas,N.R. and Spencer,P.B.
  TITLE     Polymorphic microsatellite markers in the ornate dragon lizard,
            Ctenophorus ornatus
  JOURNAL   Mol. Ecol. 9 (3), 365-366 (2000)
  MEDLINE   20200766
   PUBMED   10736034
REFERENCE   2  (bases 1 to 376)
  AUTHORS   LeBas,N.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JUN-1999) Zoology, University of Western Australia,
            Hackett Drive, Nedlands, WA 6009, Australia
FEATURES             Location/Qualifiers
     source          1..376
                     /organism="Ctenophorus ornatus"
                     /db_xref="taxon:95347"
     repeat_region   1..376
                     /note="microsatellite Co10F11"
                     /rpt_type=tandem
                     /rpt_unit=TC
BASE COUNT       70 a     96 c     88 g    122 t
ORIGIN
        1 gaatgagttc actggttttt gtgccagtta ctgtgattgg ttccagagtt gttctctctc
       61 tctctctctc tctctctctc tctctcattc actcgcttgc tcttgctttc tgtgcatgtg
      121 ttcccatcct tccctccatc cccagtgatt catggcttat aggcctcttg tccttttggc
      181 atctgctgct ccacctgata gcatcccatt tcatgaaatt tcaaggccaa agcttgggac
      241 tcaaggggtg gaccctggtg gatgtcgtag ggagtgaccc tgtgtgatgt catggggaca
      301 gggccttatg caaccatcgc aaaatggtgt gtgcgacaga caaacattgt agaggctcta
      361 tgattaactg aaaggg
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LOCUS       AF159515                 254 bp    DNA     linear   VRT 28-FEB-2001
DEFINITION  Ctenophorus ornatus microsatellite Co12E10 sequence.
ACCESSION   AF159515
VERSION     AF159515.1  GI:5639936
KEYWORDS    .
SOURCE      Ctenophorus ornatus.
  ORGANISM  Ctenophorus ornatus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Iguania; Acrodonta; Agamidae;
            Amphibolurinae; Ctenophorus.
REFERENCE   1  (bases 1 to 254)
  AUTHORS   Lebas,N.R. and Spencer,P.B.
  TITLE     Polymorphic microsatellite markers in the ornate dragon lizard,
            Ctenophorus ornatus
  JOURNAL   Mol. Ecol. 9 (3), 365-366 (2000)
  MEDLINE   20200766
   PUBMED   10736034
REFERENCE   2  (bases 1 to 254)
  AUTHORS   LeBas,N.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JUN-1999) Zoology, University of Western Australia,
            Hackett Drive, Nedlands, WA 6009, Australia
FEATURES             Location/Qualifiers
     source          1..254
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                     /db_xref="taxon:95347"
     repeat_region   1..254
                     /note="microsatellite Co12E10"
                     /rpt_type=tandem
                     /rpt_unit=GT
BASE COUNT       61 a     51 c     63 g     79 t
ORIGIN
        1 agaggacagg caggctcgta tggaagaagg cagtattctt aaattgaata cccagcttgt
       61 agggtgtgtg tgtgtgtgtg tgtgtgtgtg catgtgtagc ctgcataatt aaattgtaca
      121 ccatccagaa agtgctttaa gcacgatgag tcggtatata agcagcaggt ttttggtatt
      181 tttcatctct ttcttcctaa cattcctccc atcccacccc tgccagccaa tatttgggtt
      241 ggcagtgact agac
//

LOCUS       AF069697                 286 bp    DNA     linear   VRT 21-JUN-2002
DEFINITION  Egernia stokesii clone EST2 microsatellite.
ACCESSION   AF069697
VERSION     AF069697.1  GI:3600016
KEYWORDS    .
SOURCE      Egernia stokesii.
  ORGANISM  Egernia stokesii
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Scleroglossa; Scincomorpha; Scincoidea;
            Scincidae; Egernia.
REFERENCE   1  (bases 1 to 286)
  AUTHORS   Gardner,M.G., Cooper,S.J.B., Bull,C.M. and Grant,W.N.
  TITLE     Isolation of microsatellite loci from a social lizard, Egernia
            stokesii, using a modified enrichment procedure
  JOURNAL   J. Hered. 90 (2), 301-304 (1999)
REFERENCE   2  (bases 1 to 286)
  AUTHORS   Gardner,M.G. and Cooper,S.J.B.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JUN-1998) Evolutionary Biology Unit, South Australian
            Museum, North Terrace, Adelaide, SA 5000, Australia
FEATURES             Location/Qualifiers
     source          1..286
                     /organism="Egernia stokesii"
                     /db_xref="taxon:78585"
                     /clone="EST2"
     repeat_region   1..286
                     /note="microsatellite"
                     /rpt_type=tandem
BASE COUNT       57 a     53 c     41 g    134 t      1 others
ORIGIN
        1 gatctttatc atgttgtttt tattcacatg aaaaatctat ttgatgccag tgaaactttg
       61 agtgtgaagt actagaacaa attagaagca cngtttgcct tgacaaaagc tggaggaata
      121 gagctggaca tgggggaaat tttctttctt tctttctttc tttctttctt tctttctttc
      181 tttctttctt tctttctttc tttctttctt tctttctttc tttctttctt tctttctttc
      241 tttctttctt tctcatacag aatagtcatg ctagctcagg ggaatc
//

LOCUS       AF069699                 298 bp    DNA     linear   VRT 21-JUN-2002
DEFINITION  Egernia stokesii clone EST4 microsatellite.
ACCESSION   AF069699
VERSION     AF069699.1  GI:3600018
KEYWORDS    .
SOURCE      Egernia stokesii.
  ORGANISM  Egernia stokesii
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Lepidosauria; Squamata; Scleroglossa; Scincomorpha; Scincoidea;
            Scincidae; Egernia.
REFERENCE   1  (bases 1 to 298)
  AUTHORS   Gardner,M.G., Cooper,S.J.B., Bull,C.M. and Grant,W.N.
  TITLE     Isolation of microsatellite loci from a social lizard, Egernia
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            stokesii, using a modified enrichment procedure
  JOURNAL   J. Hered. 90 (2), 301-304 (1999)
REFERENCE   2  (bases 1 to 298)
  AUTHORS   Gardner,M.G. and Cooper,S.J.B.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JUN-1998) Evolutionary Biology Unit, South Australian
            Museum, North Terrace, Adelaide, SA 5000, Australia
FEATURES             Location/Qualifiers
     source          1..298
                     /organism="Egernia stokesii"
                     /db_xref="taxon:78585"
                     /clone="EST4"
     repeat_region   1..298
                     /note="microsatellite"
                     /rpt_type=tandem
BASE COUNT      119 a     42 c     62 g     75 t
ORIGIN
        1 gatccaatac tctggcttcc tagagtaaac ctgtgaggtt taggactgca gtttagaaag
       61 aaagaaagaa agaaagaaag aaagaaagaa agaaagaaag aaagaaagaa agaaagaaag
      121 aaagaaagaa gtgcttcctt atttccatta cttagaagta aatccctttg cattcaatac
      181 tctgacttct taccagtgta tttaggattg aagaaaaaaa gaaagaaatt cctaattttc
      241 attacttttt tgcagcgtta aaaaatgcta gggttagggt cccggggagg tcccttca
//
```

Cyclura Primer Sequences:

Primers were obtained from Catherine Malone’s Ph.D. work (with *Cyclura* and *Iguana* in the Caribbean) through personal communication with others in our lab. Most are found in her Ph.D. dissertation (2000). The sequences were discovered by screening with a tandem array of 18 GT repeats.

CCF-2/102
Forward: 5′-AGGGAGATGATTTGAGTAAC-3′
Reverse: 5′-CTAGTCAGGTGCTTGGT-3′
Annealing temperature used by the developer: 57ºC

CCF-4/70
Forward: 5′-CCTGAGGGCCCTGTACTTTC-3′
Reverse: 5′-TGATTTAACCCCCACTGGTG-3′
Not used in Ph.D. dissertation.

CCF-6/37
Forward: 5′-AGTCGCTGGTGGGATGTT-3′
Reverse: 5′-AGGCAAAAATATCYCAAAAGCAG-3′
Annealing temperature used by the developer: 55ºC

L780
Forward: 5′-GTTCACTTCCCAAGAACAGTGCA-3′
Reverse: 5′-CCTCCTCTGTAGCAGAATGTAT-3′
Annealing temperature used by the developer: 55ºC

APPENDIX F – PERMANENT IDENTIFICATION NUMBERS USED

The following table contains the identification numbers used in permanent marking procedures during work on the islet of Caamaño in the Galápagos, Ecuador during January-March, 1997, May-June, 1997, and January-February, 1998. During observations a few other numbers were recorded and assumed to have been from previous work done by others either on the same islet or with iguanas on nearby Santa Cruz that had swum over.

Table 13. Identification numbers used in permanently marking marine iguanas for observation.

Numbers used January-March, 1997

Males

0-279	(excluding all numbers with 8’s)
Exceptions	
80	included (previously marked by others)
162	excluded
161A	included along with 161
379/∞	included (right/left sides)
524/∞	included (right/left sides)

Females

1-999	(excluding all numbers with 8’s)
L0-L99	(excluding all numbers with 8’s)
V0-V16	(excluding all numbers with 8’s)
Exceptions	
8	included
18	included
28	included

Numbers used May-June, 1997

Hatchlings

1-602	(excluding all numbers with 8’s)
Exceptions	
124	excluded
123	included (line below number)
331	excluded
329	included (line below number)
1	does not have “|” mark at base of tail, all others do

Numbers used January-February, 1998

Males

290-297

Notation

293 was previously marked 196

Hatchlings (from previous season)

603-617	(excluding all numbers with 8’s)

APPENDIX G – BASIC MOLECULAR MARKER DEVELOPMENT METHODS

Extraction of DNA from Blood Sample

Common phenol-chloroform DNA extraction was found to produce the most consistent and pure DNA from blood samples. Many of the samples contained degraded DNA and produced only a small quantity of long (high molecular weight) high quality DNA. However, sufficient DNA was extracted in high quality in order for microsatellite development to proceed. The standard phenol-chloroform extraction method used follows, however, since a large quantity of high quality DNA was needed for library construction the recipe was multiplied by 5 times.

- Use 0.01-0.50 g or fresh tissue and place in a 1.5 ml microcentrifuge tube
- Add 200-400 µl lysis buffer and macerate tissue, adding more lysis buffer if sample is too viscous
- Microfuge for 3 min. at 10,000 rpm
- Remove supernatant and place in a new microfuge tube
- Add equal volume of buffer-equilibrated phenol and invert gently
- Centrifuge for 10 min. at 10,000 rpm
- Remove upper aqueous layer with a pipette and place in a new microfuge tube
- Add equal volume of phenol:chloroform (1:1) and invert gently
- Centrifuge for 5 min. at 10,000 rpm
- Remove upper aqueous layer with a pipette and place in a new microfuge tube
- Add equal volume of chloroform and invert gently

- Centrifuge for 3 min. at 10,000 rpm
- Remove upper aqueous layer with a pipette and place in a new microfuge tube
- Add ½ volume 7.5 M NH_4OAc (ammonium acetate)
- To the new volume add enough ethanol (100% / 200 proof) to make the solution 66% ethanol and invert gently, then wait as long as 10 min. for all samples to develop precipitate
- Centrifuge for 10 min. at 10,000 rpm
- Carefully decant liquid so as not to dislodge pellet
- Finger flick to dislodge pellet and then fill each tube with 70% ethanol
- Let stand for 10 min.
- Centrifuge for 10 min. at 10,000 rpm
- Decant off liquid and use a Kimwipe to swab dry remaining fluid – ensuring the pellet is not touched
- Air dry pellet
- Add 100 µl sterile TE to each sample and allow the DNA to dissolve
- Freeze at -20ºC until needed (or if needed quite frequently store at 4ºC)

Preparation of Partial Genomic Library

A general overview of the process in graphical form is shown in Figure 75. Specific procedures are outlined below.

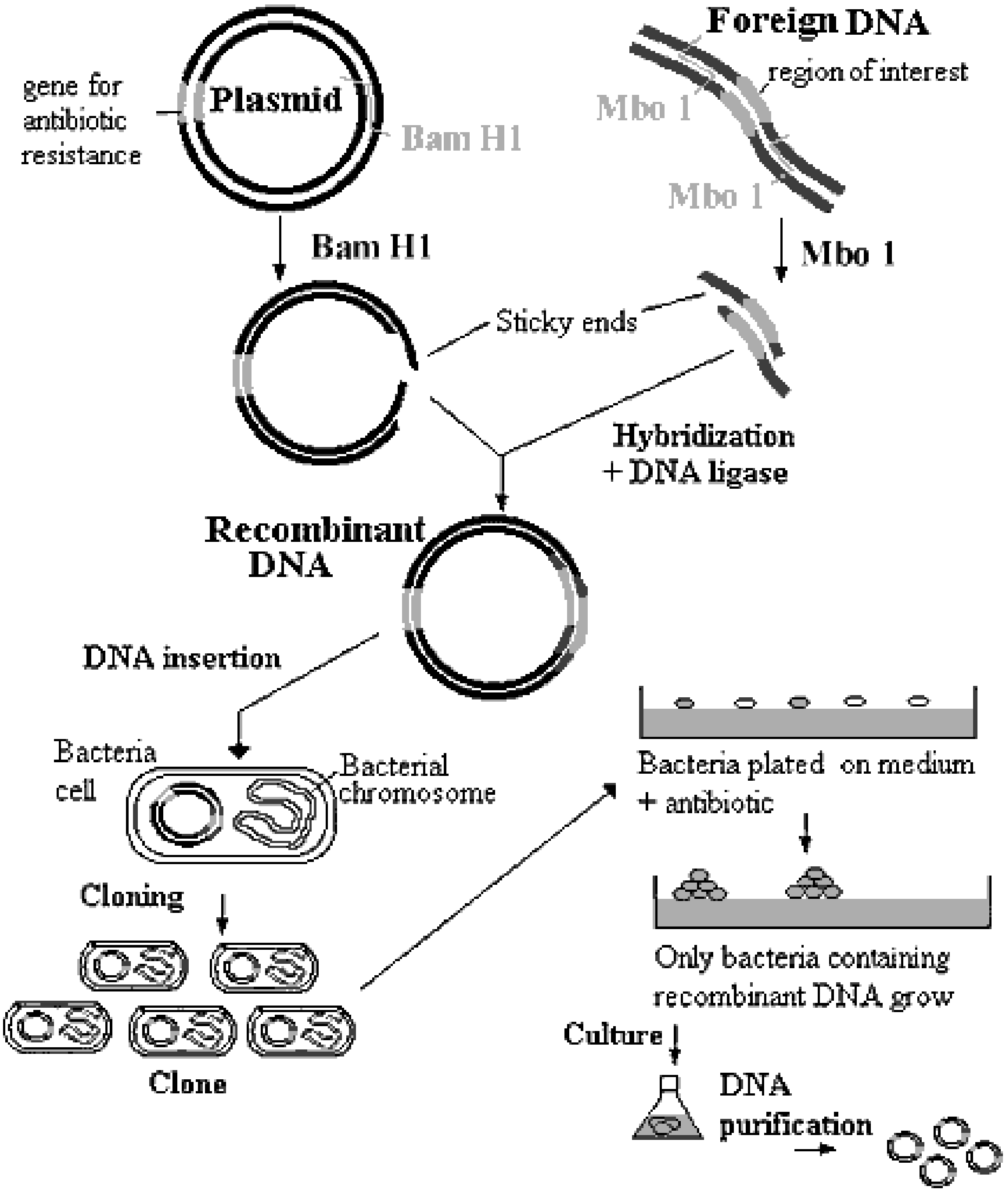


Figure 75. Overview of partial genomic library construction method used.

DNA Fragments and Plasmid/Vector Ligation:

Purified DNA was restricted (cut) using *Mbo* 1 (which cuts at the same position as *Sau* 3A1).

- 200 µl DNA (100-300 ng/µl)
- 25 µl 10x Buffer K (from kit)
- 5 µl *Mbo* 1
- adjust volume to 250 µl with PCR grade water
- Digest 3 hr. at 37^{o}C (with occasional mixing/tapping)
- Inactivated enzyme by incubating at 80^{o}C for 15 min.

Plasmid (pUC19) was cut with *Bam* H1.

- 2 µl pUC19 (measured by UV 260/280 at 200 ng/µl)
- 2 µl 10x Buffer K (from kit)
- 1 µl *Bam* H1
- 15 µl PCR grade water
- Digested 90 min. at 30^{o}C
- Inactivated enzyme by incubating at 80^{o}C for 15 min.
- Separated out and saved 10 µl to use as a ligation control later on

Plasmid (pUC19) was dephosphorylated using Shrimp Alkaline Phosphatase (SAP).

- 10 µl restricted pUC19
- 1.2 µl 10x buffer (from kit)
- 1 µl SAP
- Incubated 10 min. at 37°C
- Inactivated enzyme by incubating at 65°C for 15 min.

Restricted pUC19 samples were extracted using phenol/chloroform/isoamylalcohol.

- 12 µl pUC19 sample
- 200 µl TE
- 200 µl phenol/chloroform/isoamylalcohol (25:24:1)
- Vortexed (shook VERY well) 10 sec. and then centrifuged at 21,000g for 1 min.
- Removed top aqueous layer (containing DNA) to a new microfuge tube
- Added 20 µl 4 M LiCl and mixed
- Added 500 µl 100% ethanol (non-denatured) and inverted several times
- Put into -20°C overnight
- Centrifuged at 21,000g for 1 min.

- Poured off ethanol and allowed pellet of DNA to dry
- Re-suspended plasmid in TE and quantified by UV (260/280)
- Diluted plasmid DNA concentration with TE to 100 ng/µl

Restricted DNA was extracted using phenol/chloroform/isoamylalcohol.

- 250 µl restricted DNA sample
- 200 µl TE
- 200 µl phenol/chloroform/isoamylalcohol (25:24:1)
- Vortexed (shook VERY well) 10 sec. and then centrifuged at 21,000g for 1 min.
- Removed top aqueous layer (containing DNA) to a new microfuge tube
- Added 40 µl 4 M LiCl and mixed
- Added 1 ml 100% ethanol (non-denatured) and inverted several times
- Put into -20^{o}C for a few hours to precipitate DNA
- Centrifuged at 21,000g for 4 min.
- Poured off ethanol, added 1 ml 70% ethanol, and inverted several times
- Centrifuged at 21,000g for 4 min.
- Poured off ethanol and allowed pellet of DNA to dry
- Re-suspended DNA in 20 µl TE and quantified by UV (260/280)

Restriction and extraction products were checked on a 4% polyacrylamide gel in order to ensure the DNA for insertion had been chopped up and that the plasmids had been cut, preparing them for insertion of the chopped up DNA fragments.

Ligation of Plasmid/Vector and DNA Fragments (inserting fragments into circular DNA):

- 1 µl cut pUC19 plasmid DNA (measured at 200 ng/µl)
- 2 µl digested DNA (100 ng/µl, 2x pUC19 to match DNA amounts)
- 1 µl T4 DNA ligase
- 1 µl 10x T4 DNA ligase buffer (from kit)
- 5 µl PCR grade water
- Incubated at 16^{o}C overnight

Preparation of Cells (*E. coli*) for Insertion of DNA Fragments:

Prepared overnight cultures for inoculation of growth media.

- Sterile toothpick was used to remove 2-5 colonies of *E. coli* from a petri dish (previously spread and grown overnight followed by storage at 4^{o}C)
- Toothpick was placed into sterile test tube containing 4 ml LB broth
- Tubes containing toothpicks were incubated at 37^{o}C overnight shaking at 200 rpm

Preparation of competent cells from overnight cultures (so plasmid DNA can be inserted).

- Flasks containing 50 ml LB broth were inoculated with 0.5 ml fresh overnight culture
- Incubated flasks at 37^oC shaking at 190 rpm until spectrophotometer A_{600} (absorbance at a wavelength of 600 nm) reading was between 0.25-0.4
- Transferred cell culture to 50 ml centrifuge tubes and cooled cells for 5 min. on ice
- Centrifuged at 3500 rpm for 10 min. at 4^oC (Beckman benchtop model)
- Poured off liquid and gently resuspended cells in 20 ml sterile ice-cold 5 M $CaCl_2$
- Incubated on ice for 30 min.
- Centrifuged at 3500 rpm for 10 min. at 4^oC
- Poured off liquid and gently resuspended in 4 ml sterile ice-cold $CaCl_2$

Transformation of Competent Cells (inserting the DNA):

- Diluted ligation results by mixing 5 µl ligation solution with 10 µl Tris-HCl buffer

- Put 200 µl competent cells and 12 µl diluted ligation result (from above) into a sterile glass tube (a lesser quantity, 5 µl ligated solution, recommended in protocol did not produce good results)
- Incubated on ice for 45 min.
- Incubated in a water bath at 42°C for 3 min. (with no shaking)
- Chilled in an ice bath for 1 min.
- Removed from the ice bath and added 800 µl of 37°C LB broth
- Incubated at 37°C for 60 min. with shaking every 10-15 min.

Blue/white colony selection was used to determine which bacterial colonies grown thinly on nutrient rich growth medium containing ampicillin in petri dishes contained inserted DNA fragments. The plasmid (or vector) used (pUC19) inserted into the bacteria (*E. coli*) during transformation contains a gene called LacZ. The LacZ gene codes for the production of an enzyme called beta-galactosidase. This enzyme is made up of two fragments, alpha and omega. When the two fragments are associated they form a functional enzyme. Normally beta-galactosidase metabolizes galactose producing two products, lactose and glucose. Beta-galactosidase converts other substrates such as X-Gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) into a colored product. X-Gal is a colorless modified galactose sugar, however, when it is metabolized by beta-galactosidase a bright blue product is produced.

In order for the gene to be actively transcribed from the DNA and for the enzyme to be produced, an activator called IPTG (isopropyl-[beta]-D-thiogalactopyranoside) must be added. Both X-Gal and IPTG are delivered to the bacteria through the growth medium (generally a supplemented and enriched bacto-agar blend).

Within the LacZ gene there are multiple cloning sites where the plasmid may be cut and DNA may be added. This produces a plasmid with foreign DNA located within the LacZ gene. When transcription of the gene is activated by IPTG the foreign DNA that has been inserted is transcribed as well. When the gene is later translated into the enzyme the inserted DNA is translated as well. Because of its location within the enzyme the foreign DNA's translated protein product disrupts activity and function of the enzyme. The disrupted enzyme activity is observed as a white bacterial colony. (If the enzyme is functioning fully each colony is a bright blue color.) Very small inserts of foreign DNA may lead to light blue colonies.

It is also important to note that the plasmid inserted into the bacteria (pUC19) contains a gene conferring ampicillin resistance. This means that only bacteria that actually have had plasmids inserted into them will survive on the growth medium containing ampicillin. So all bacteria colonies seen growing contain an inserted plasmid, however, only white colonies have plasmids with DNA (the partial genomic library) inserted into them.

Plating Out Transformed Cells (detection of cells with inserted plasmids):

- Prepared LB/ampicillin/X-gal/IPTG plates

  - Poured LB agar/ampicillin plates (10 cm) containing 1 ml/l ampicillin solution (added after autoclaved LB agar had cooled to “touchable” temperature) and cooled
  - Mixed a 1:1 solution of X-gal and IPTG solutions
  - Spread 50 µl of 1:1 solution evenly on the surface of the cool plates
  - Allowed to soak in at 37°C for 4 hr.
- Spread 200 µl of transformed cells over each plate
- Allowed spread cell solution to soak in before turning plates over
- Incubated at 37°C until colonies were distinct and blue/white detection system was visible

Screening of Partial Genomic Library

An overview of the non-radioactive screening procedure used is shown in Figure 76. The specific steps and procedures are outlined below:

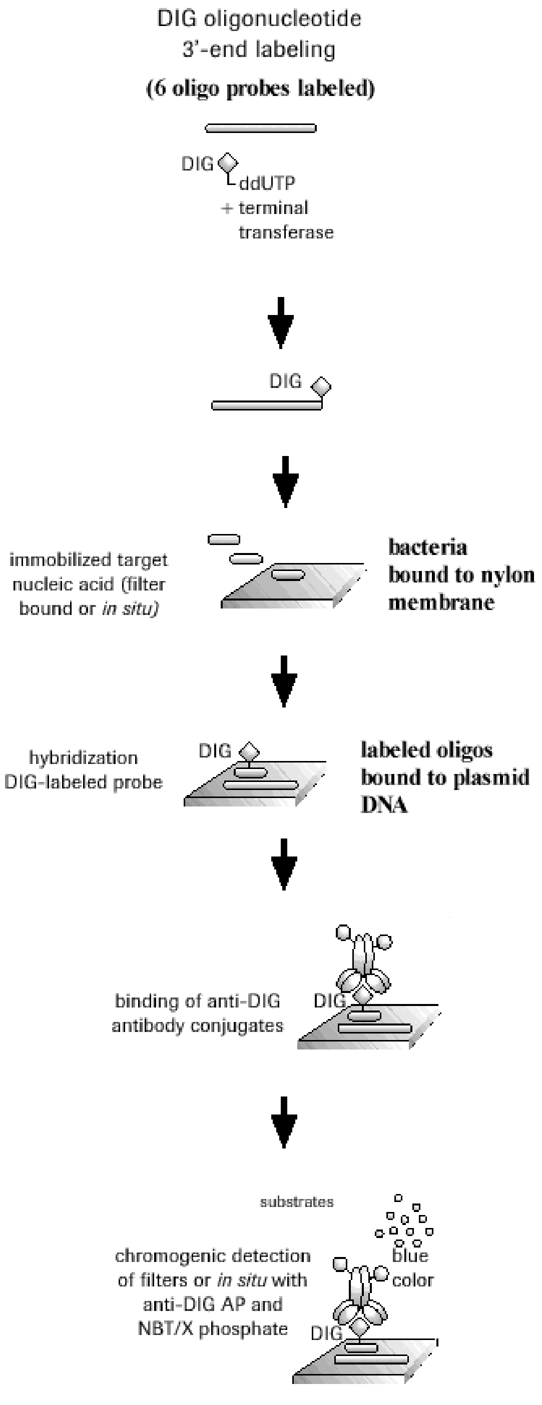


Figure 76. Overview of non-radioactive colony screening method used during microsatellite marker development.

Colony Growth and Non-Radioactive Detection:

- Poured LB agar/ampicillin plates (10 cm x 10 cm), but with no IPTG or X-gal (see page 209)
- Using sterile toothpicks patched over (touching toothpick to original bacterial colony, to a labeled plate, and then again to a replicate plate) colonies that had plasmids (those that were white rather than blue) onto plates with grids of 26 x 26
- Incubated at 37°C until there was substantial bacterial growth
- Transferred to 4°C for storage

Oligonucleotide Labeling

- Oligonucleotides synthesized by Genosys based upon the recommendations found in the document "*Microsatellite markers: Isolation with non-radioactive probes and amplification* (Version of 12/1996)" written by Arnaud Estoup and Julie Turgeon:

$(TC)_{10}$	$(TG)_{10}$	$(CAC)_5CA$
$CT(CCT)_5$	$(TGTA)_6TG$	$CT(ATCT)_6$

- Rehydrated oligonucleotides containing repeat sequences in TE to 100 pmol/µl

- Labeled oligonucleotides according to the protocol provided with the DIG Oligonucleotide 3'-End Labeling Kit purchased from Roche Molecular Biochemicals

Hybridization and Detection of DIG Labeled Probes

- Cut positively charged nylon membranes to fit petri dishes containing grids of bacterial colonies
- Labeled membranes with a ballpoint pen along the top edge in multiple places
- Carefully placed membranes on surface of petri dishes for a few minutes (being sure not to drag at any time)
- Removed membrane and allowed to air dry (usually 10-30 min.)
- Treated each membrane by the following four soaking (not submerging) steps:
    - 10% SDS - 3 min.
    - Denaturing solution - 5 min.
    - Neutralizing solution - 5 min.
    - 2x SSC - 5 min.
- Let membrane air dry (approximately 30 min.)
- Crosslinked (bound) DNA to membrane in a Stratagene UV Stratalinker on the “automatic” setting (120,000 µJ)
- Pre-heated hybridization oven, rollers, and PerfectHyb Plus hybridization buffer (from Sigma) to 46^{o}C

- Threaded a string through the top labeled portion of the membranes (to hold them in place so they wouldn't slide around - with the string pinched between the cover and glass of the roller)
- Placed them in the warmed rollers containing warmed buffer and pre-hybridized for 10-15 min.
- Added 2 µl of each labeled oligonucleotide (12 µl total) to each hybridization tube
- Hybridization at 46^{o}C continued overnight
- Washed with low stringency wash buffer at room temperature for 5 min.
- Washed with high stringency wash buffer two times for 20 min. at room temperature

  Note: Because bacterial colonies were lifted on membranes from plates that had been prepared for IPTG/X-gal blue/white screening there were many blue spots on the membranes even before the colorimetric procedure was started. To account for this the membranes were photographed both before and after the colorimetric procedure. (It is a good idea not to use IPTG/X-gal blue/white screening on the plates that will be used for colorimetric screening later!)

- Colorimetric detection of DIG labeled nucleic acids was performed following the instructions provided with the DIG Nucleic Acid Detection Kit purchased from Roche Molecular Biochemicals

Selection of Colonies for Plasmid Extraction

Colonies that had a very dark purple color were considered to be positive (containing iguana DNA within the plasmid as well as containing a microsatellite repeat sequence within the inserted DNA). Positive colonies were identified and then grown up in overnight cultures for plasmid DNA extraction.

Overnight cultures contained 4 ml sterile autoclaved LB broth with a sterile toothpick that had been touched to a selected positive bacteria colony and added to the broth (using sterile procedure). Incubation was at 37^{o}C overnight on an orbital shaker at 200 rpm.

Plasmid DNA extraction from overnight cultures was done using QIAGEN's QIAprep Spin Miniprep Kit. Approximate DNA concentration was determined by agarose gel electrophoresis with a known quantitative DNA ladder utilizing the “spot density” function included with the Alpha Innotech IS-1000 Digital Imaging System used for documentation.

APPENDIX H – INSTRUCTION MANUAL FOR DIVERSION

(Population Simulation Program)

The following is the instruction manual created for the population simulation program Diversion. Due to use of space the full program source is not listed here, however, it is included on the CD/DVD included with this dissertation. The program may be freely modified to meet whatever needs the user might have and may be freely distributed. Under no circumstances may any charge (materials, labor, or otherwise) ever be made for it or any modified version. Reference must always be included for the original author.

Diversion

"Genocidal tendencies" . . . must be defined.

Created by:

Jeffrey A. Sonnentag

A Foxy Software Production

With support from:

Loma Linda University, Natural Sciences Department

"To Make Man Whole"

Mónica X. Freire's fund for the poor

Copyright © 2002 Jeffrey A. Sonnentag, Foxy Software

Diversion

Purpose

The original purpose of this population simulation program was to test out the idea that frequent periodic population crashes and booms might lead to coincidental association of phenotypes with genotypes (microsatellite repeat length in this instance). With that in mind it was created with flexibility in order to test multiple hypotheses and scenarios. However, flexibility is not unlimited and several assumptions are made during processing. These limitations are explained below.

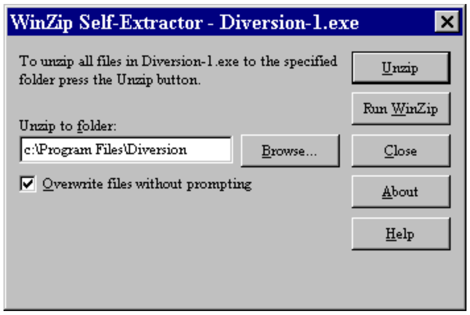
The following written description along with diagrammatic flowcharts is intended to fully explain how the program works so that the user might decide intelligently if it meets a current need or might easily be modified to fulfill some related purpose. To this end the full original program code (Diversion.x), written in XBasic 6.2.2 (which can be compiled to run on both Windows and Linux operating systems) is included.

Overview

Installation & Removal:

The following files are included in the self-extracting ZIP archive:

- Diversion.exe (main executable)
- xb.dll (dynamic linked library of functions)
- Diversion.hlp (help file)
- Diversion.doc / Diversion.pdf / Diversion.txt (documentation)
- DiversionInfo.gif (program information graphic)
- default.div (sample variable file)
- Diversion.x (original program written in XBasic, in simple text format)


WinZip Self-Extractor - Diversion-1.exe

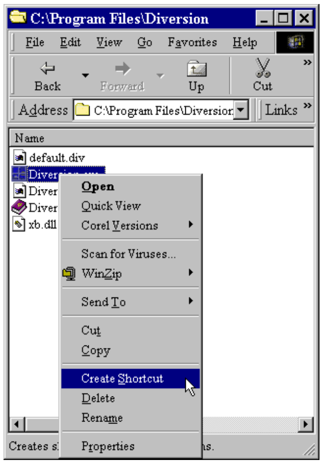
To unzip all files in Diversion-1.exe to the specified folder press the Unzip button.

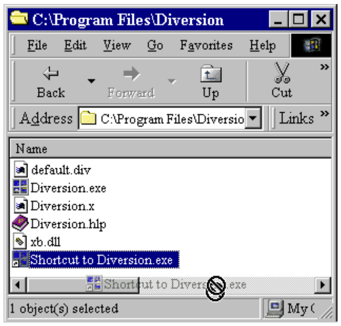
Unzip to folder: c:\Program Files\Diversion

☑ Overwrite files without prompting

Windows:

To install the program under Windows (95 and newer) simply run the self-extracting archive (Diversion-1.exe) and when prompted for a location to "unzip" the program enter in a directory you can easily find (such as c:\Diversion or c:\Program Files\Diversion). After the files have been extracted close the self-extractor window.





To create a “shortcut” to the program for faster access find the folder or directory containing the installed program and right-click on the main executable (Diversion.exe). Select “Create Shortcut” and when the shortcut appears in the file listing drag it to the location you want (such as the desktop or down to the shortcut bar beside the “Start” button). If the only shortcut desired is to be placed in the shortcut bar beside the “Start” button it can be placed there by simply “dragging” the main executable (Diversion.exe) from the file listing to that bar. A shortcut is created while the original file is left in the folder/directory.

To remove the program, delete the folder or directory that was created during setup. All components used by the software are contained in the folder. However, remember to check for data files that may have been saved in the same directory.

Program Source and Distribution:

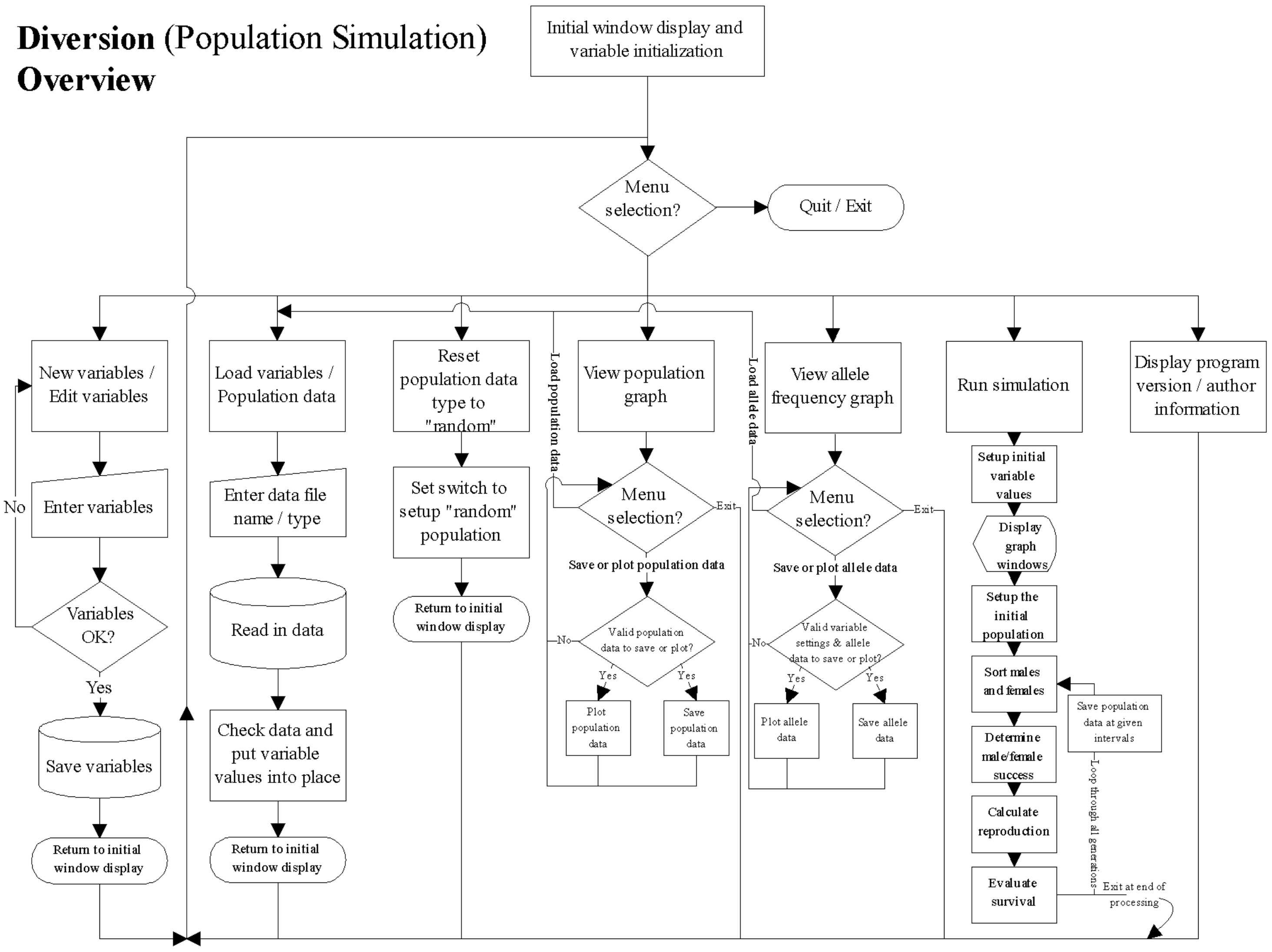
The original source written in XBasic is freely available and may be modified to meet individual purposes. Any and all modifications should be clearly noted if further distribution of a modified version is made.

How the Program Works:

The following are flowcharts describing the processing performed by the program. They are provided for both reference and as helpful reminders of some limitations. Viewing the text file (Diversion.x) containing the full program code in XBasic may be useful for clarification of some processes.

First (on the next page) is an overview of the entire program with the main processes and how they interrelate with each other highlighted. The second flowchart is an overview of one specific process central to the whole program, running the population simulation itself. The flowcharts following that procedural overview are a further breakdown of the simulation’s calculations. Each process is located within a separate subroutine within the simulation module and is repeated for each generation in turn.

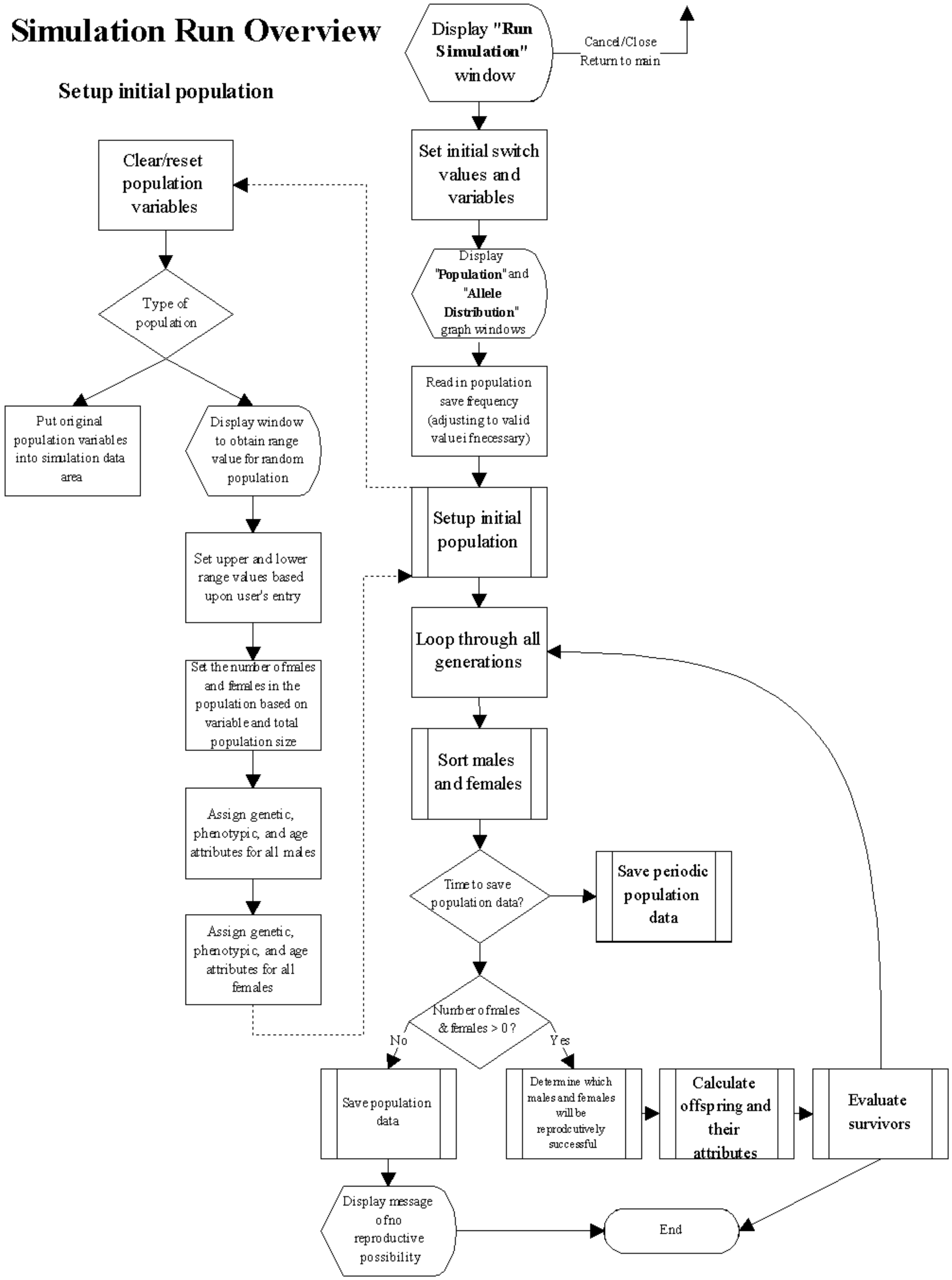
Diversion (Population Simulation) **Overview**



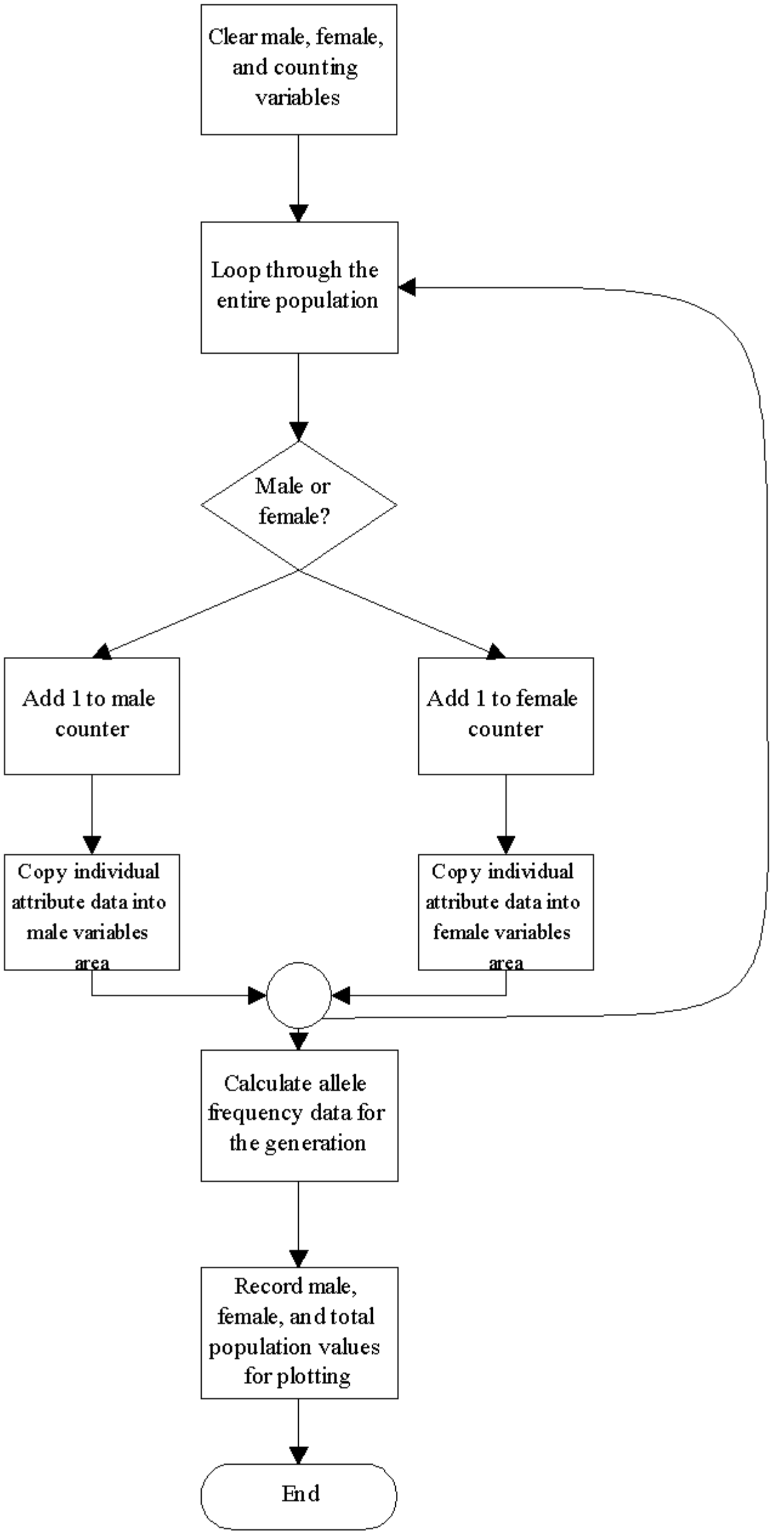
Initial window display and variable initialization
Menu selection?
Quit / Exit
New variables / Edit variables
Enter variables
No
Variables OK?
Yes
Save variables
Return to initial window display
Load variables / Population data
Enter data file name / type
Read in data
Check data and put variable values into place
Return to initial window display
Reset population data type to "random"
Set switch to setup "random" population
Return to initial window display
Load population data
View population graph
Menu selection?
Exit
Save or plot population data
No
Valid population data to save or plot?
Yes
Yes
Plot population data
Save population data
Load allele data
View allele frequency graph
Menu selection?
Exit
Save or plot allele data
No
Valid variable settings & allele data to save or plot?
Yes
Yes
Plot allele data
Save allele data
Run simulation
Setup initial variable values
Display graph windows
Setup the initial population
Sort males and females
Save population data at given intervals
Determine male/female success
Calculate reproduction
Evaluate survival
Loop through all generations
Exit at end of processing
Display program version / author information

Simulation Run Overview

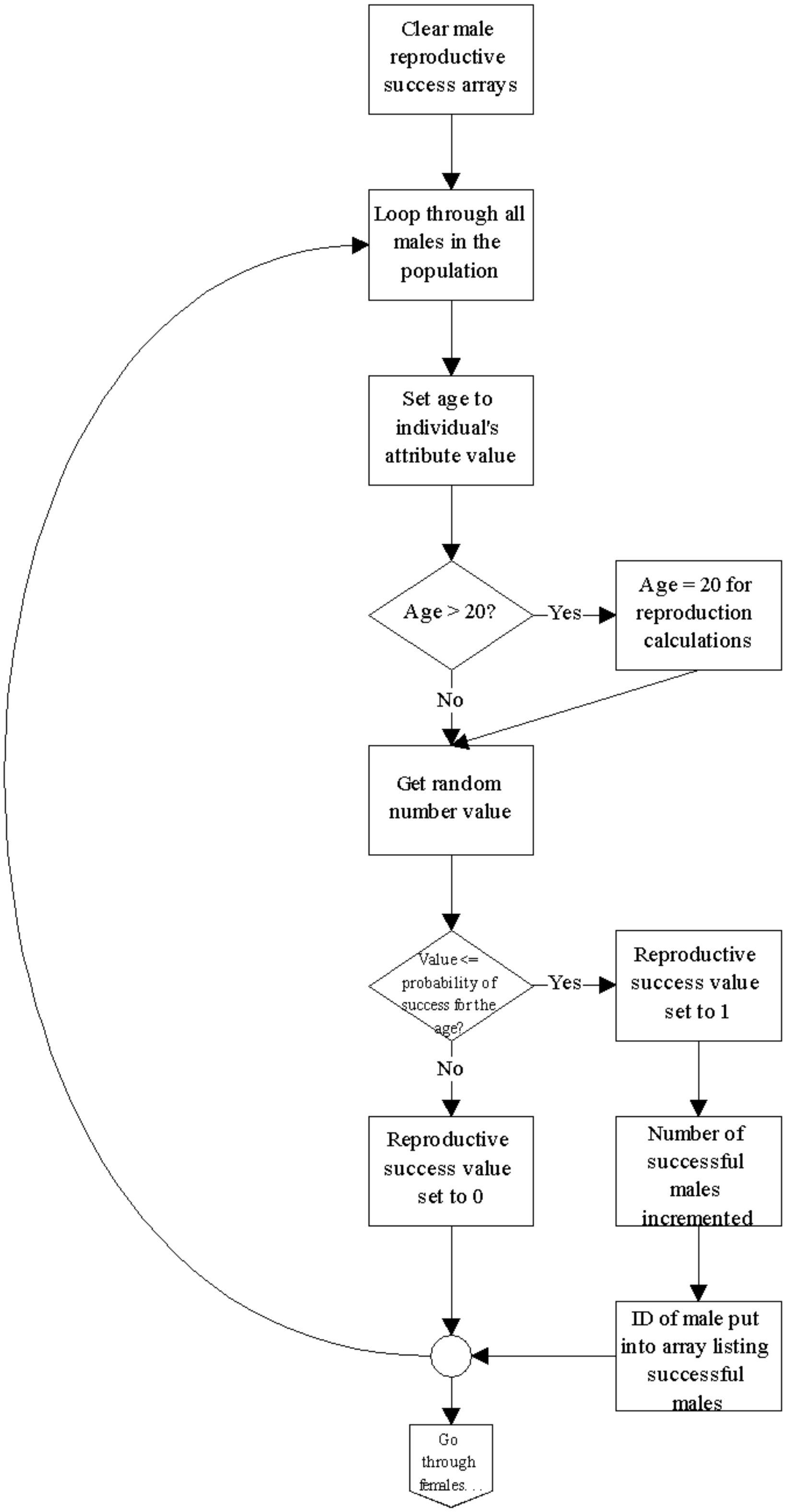
Setup initial population


Display "Run Simulation" window
Cancel/Close
Return to main
Set initial switch values and variables
Display "Population" and "Allele Distribution" graph windows
Read in population save frequency (adjusting to valid value if necessary)
Setup initial population
Loop through all generations
Sort males and females
Time to save population data?
Save periodic population data
Number of males & females > 0 ?
No
Yes
Save population data
Display message of no reproductive possibility
End
Determine which males and females will be reprodcutively successful
Calculate offspring and their attributes
Evaluate survivors
Clear/reset population variables
Type of population
Put original population variables into simulation data area
Display window to obtain range value for random population
Set upper and lower range values based upon user's entry
Set the number of males and females in the population based on variable and total population size
Assign genetic, phenotypic, and age attributes for all males
Assign genetic, phenotypic, and age attributes for all females

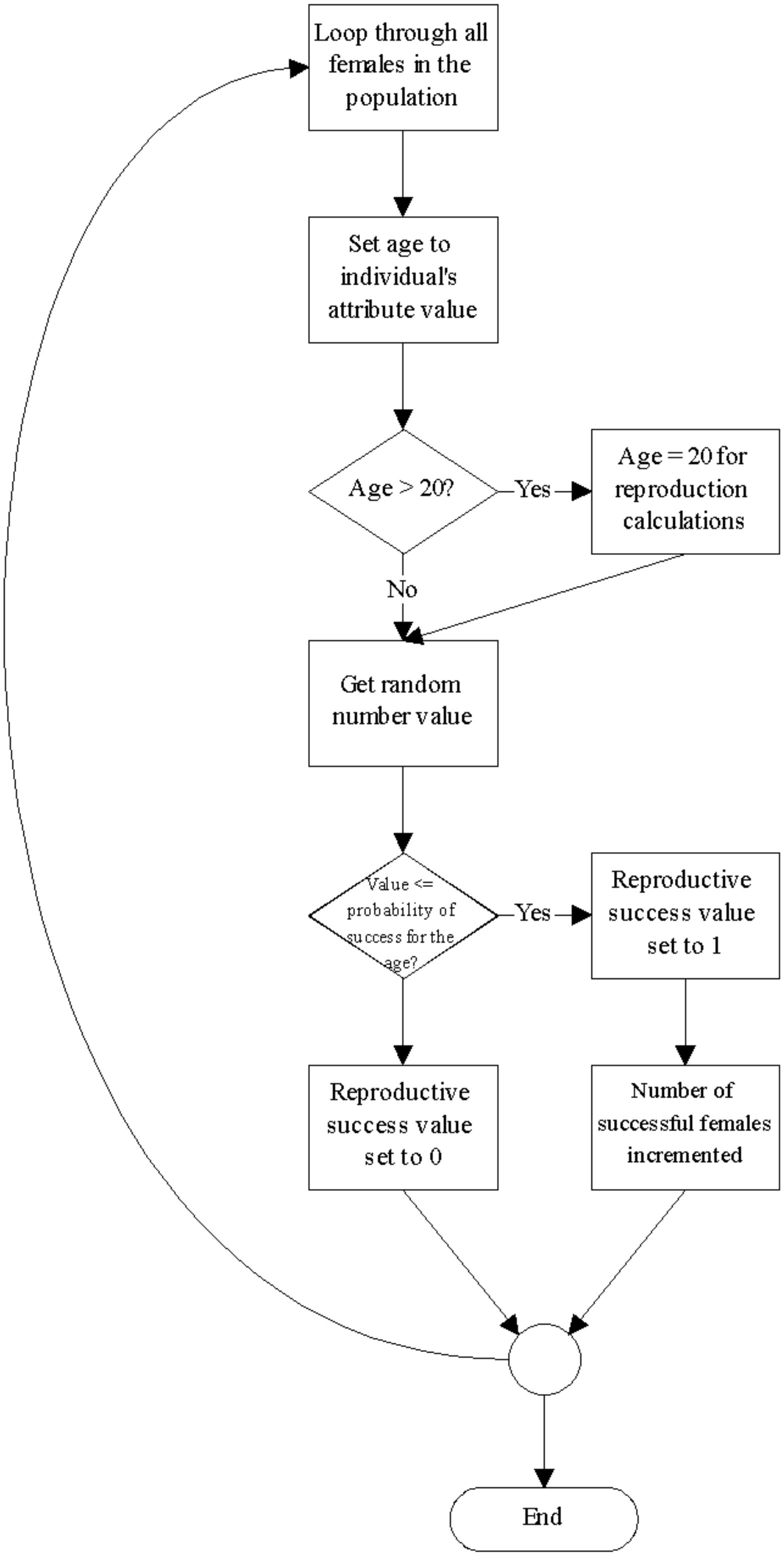
Sort males and females


Clear male, female, and counting variables
Loop through the entire population
Male or female?
Add 1 to male counter
Add 1 to female counter
Copy individual attribute data into male variables area
Copy individual attribute data into female variables area
Calculate allele frequency data for the generation
Record male, female, and total population values for plotting
End

Male / Female Reproductive Success

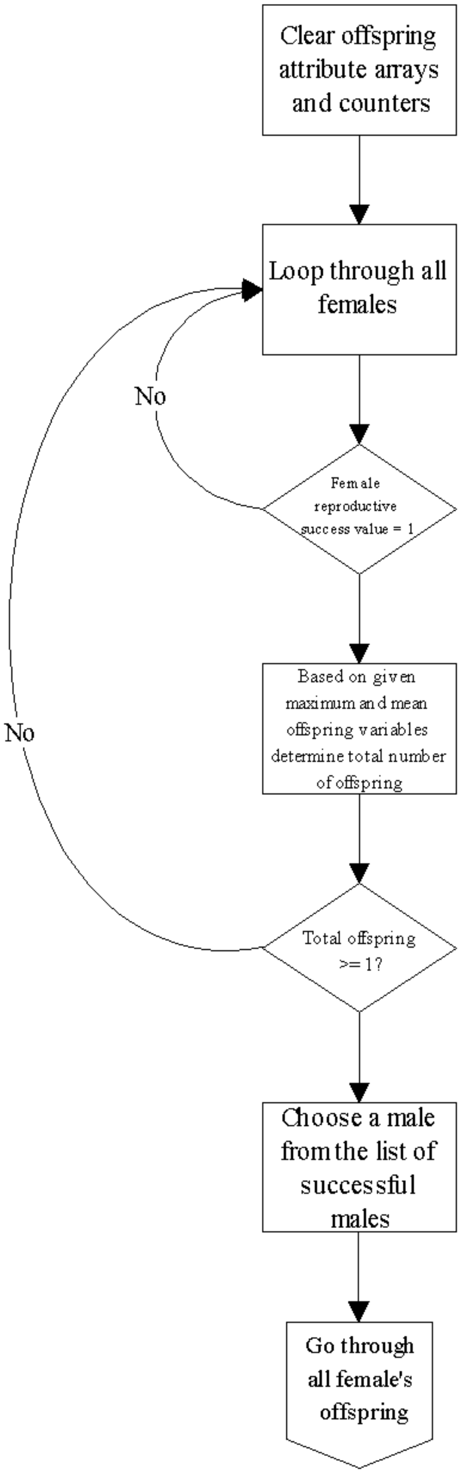

Clear male reproductive success arrays
Loop through all males in the population
Set age to individual's attribute value
Age > 20?
Yes
Age = 20 for reproduction calculations
No
Get random number value
Value <= probability of success for the age?
Yes
Reproductive success value set to 1
No
Reproductive success value set to 0
Number of successful males incremented
ID of male put into array listing successful males
Go through females. . .

Male / Female Reproductive Success (cont.)


Loop through all females in the population
Set age to individual's attribute value
Age > 20?
Yes
Age = 20 for reproduction calculations
No
Get random number value
Value <= probability of success for the age?
Yes
Reproductive success value set to 1
Reproductive success value set to 0
Number of successful females incremented
End

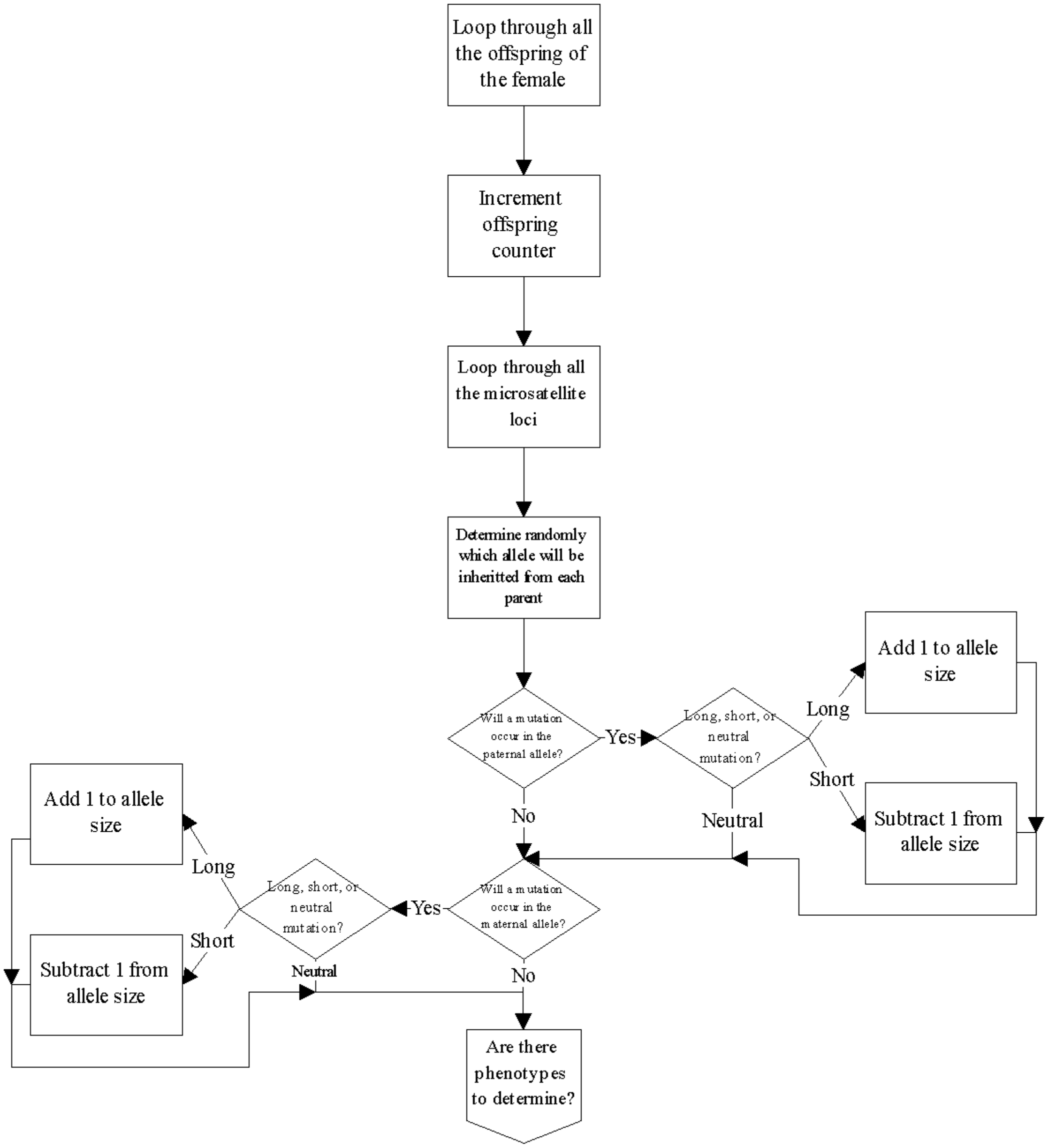
Reproduction

(Calculation of offspring and their attributes)


Clear offspring attribute arrays and counters
Loop through all females
No
Female reproductive success value = 1
Based on given maximum and mean offspring variables determine total number of offspring
No
Total offspring >= 1?
Choose a male from the list of successful males
Go through all female's offspring

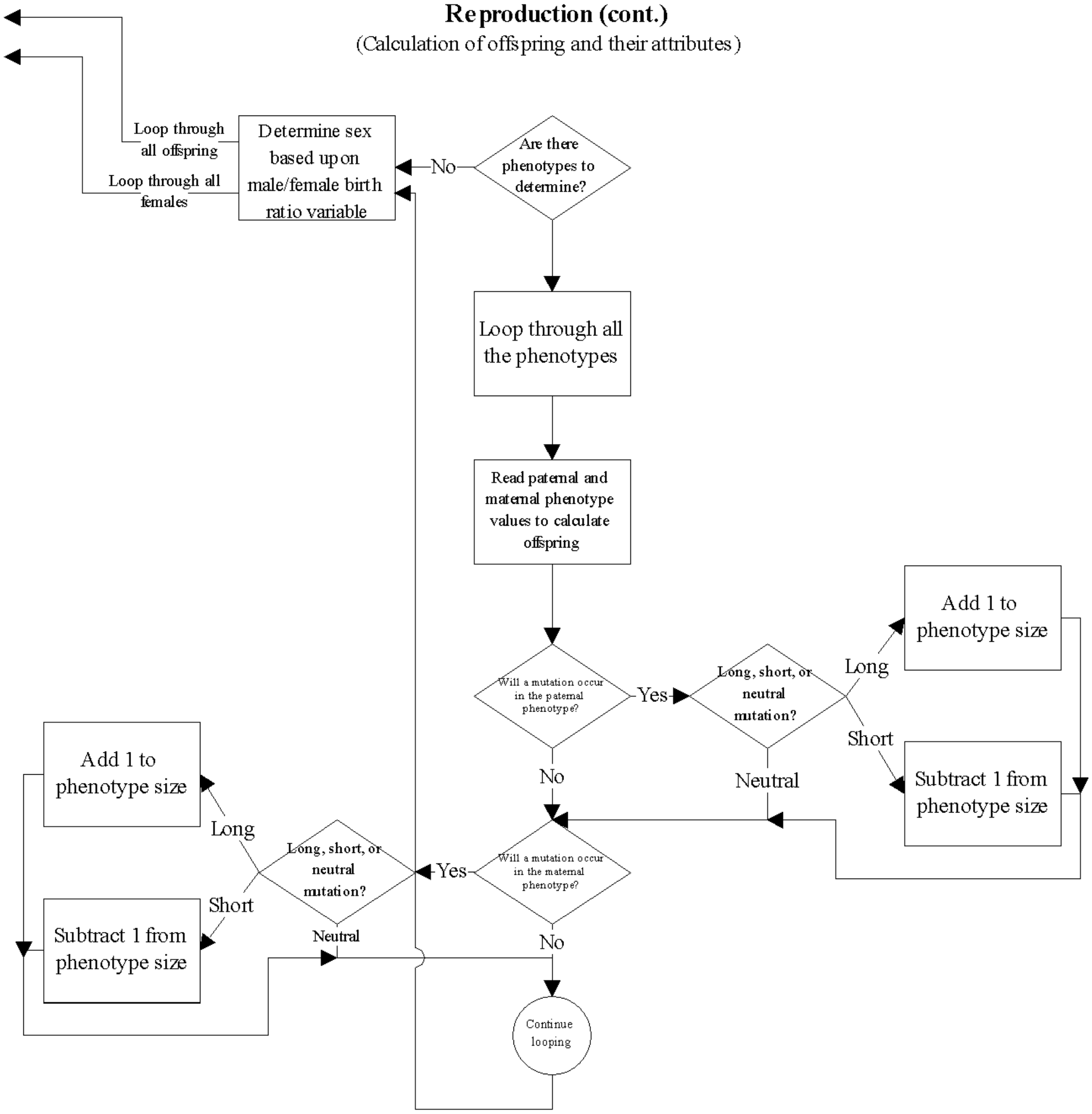
Reproduction (cont.)

(Calculation of offspring and their attributes)


Loop through all the offspring of the female
Increment offspring counter
Loop through all the microsatellite loci
Determine randomly which allele will be inheritted from each parent
Will a mutation occur in the paternal allele?
Yes
Long, short, or neutral mutation?
Long
Add 1 to allele size
Short
Subtract 1 from allele size
Neutral
No
Will a mutation occur in the maternal allele?
Yes
Long, short, or neutral mutation?
Long
Add 1 to allele size
Short
Subtract 1 from allele size
Neutral
No
Are there phenotypes to determine?

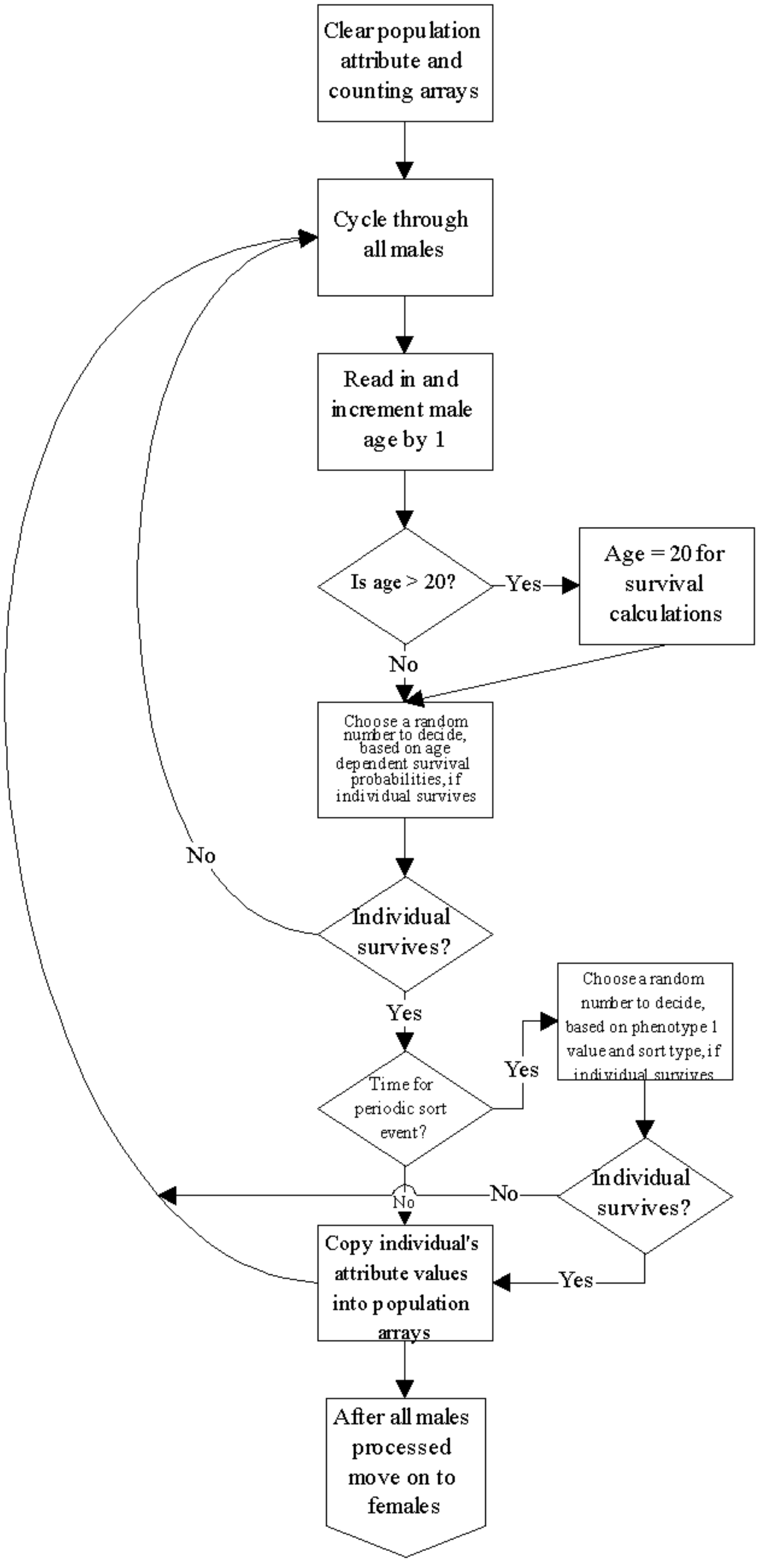
Reproduction (cont.)

(Calculation of offspring and their attributes)

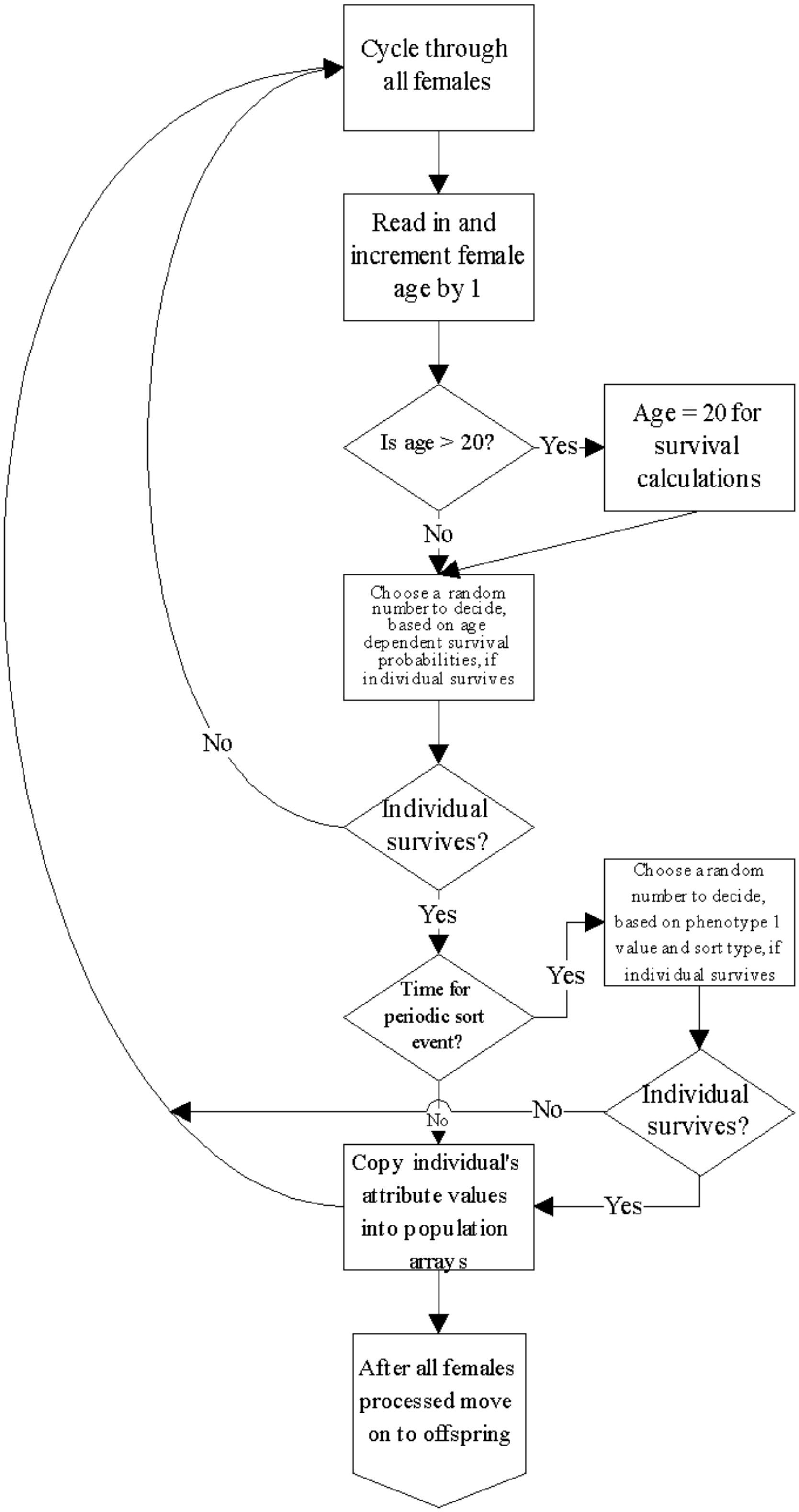


Loop through all offspring
Loop through all females
Determine sex based upon male/female birth ratio variable
No
Are there phenotypes to determine?
Loop through all the phenotypes
Read paternal and maternal phenotype values to calculate offspring
Will a mutation occur in the paternal phenotype?
Yes
Long, short, or neutral mutation?
Long
Add 1 to phenotype size
Short
Subtract 1 from phenotype size
Neutral
No
Will a mutation occur in the maternal phenotype?
Yes
Long, short, or neutral mutation?
Long
Add 1 to phenotype size
Short
Subtract 1 from phenotype size
Neutral
No
Continue looping

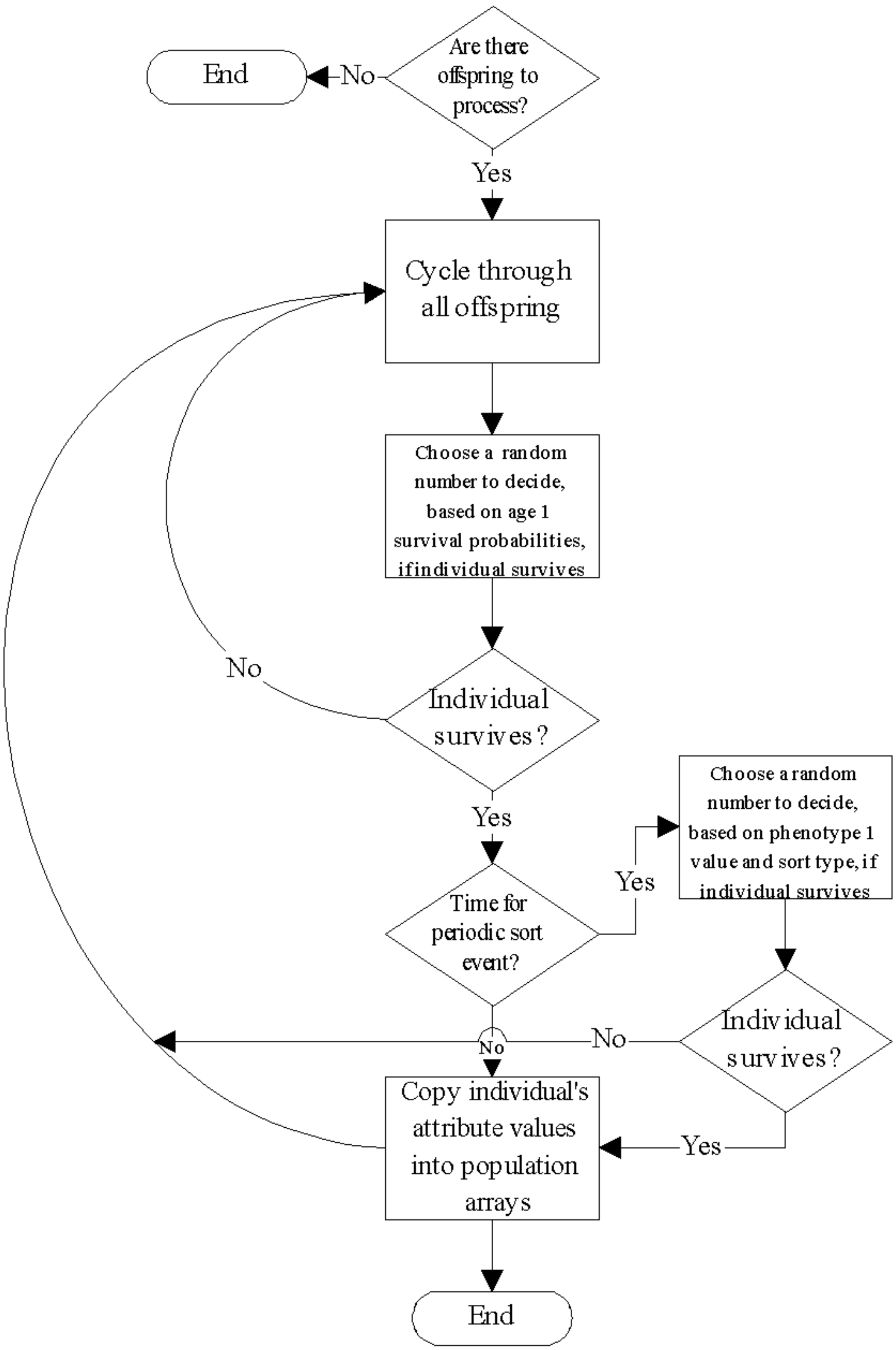
Survivors


Clear population attribute and counting arrays
Cycle through all males
Read in and increment male age by 1
Is age > 20?
Yes
Age = 20 for survival calculations
No
Choose a random number to decide, based on age dependent survival probabilities, if individual survives
No
Individual survives?
Yes
Time for periodic sort event?
Yes
Choose a random number to decide, based on phenotype 1 value and sort type, if individual survives
Individual survives?
No
No
Yes
Copy individual's attribute values into population arrays
After all males processed move on to females

Survivors (cont.)


Cycle through all females
Read in and increment female age by 1
Is age > 20?
Yes
Age = 20 for survival calculations
No
Choose a random number to decide, based on age dependent survival probabilities, if individual survives
No
Individual survives?
Yes
Time for periodic sort event?
Yes
Choose a random number to decide, based on phenotype 1 value and sort type, if individual survives
Individual survives?
No
No
Yes
Copy individual's attribute values into population arrays
After all females processed move on to offspring

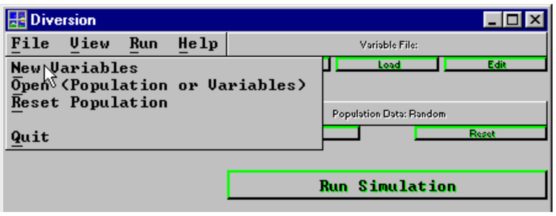
Survivors (cont.)

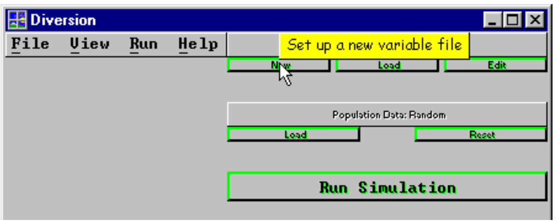

Are there offspring to process?
No
End
Yes
Cycle through all offspring
Choose a random number to decide, based on age 1 survival probabilities, if individual survives
Individual survives?
No
Yes
Time for periodic sort event?
Yes
Choose a random number to decide, based on phenotype 1 value and sort type, if individual survives
Individual survives?
No
No
Yes
Copy individual's attribute values into population arrays
End

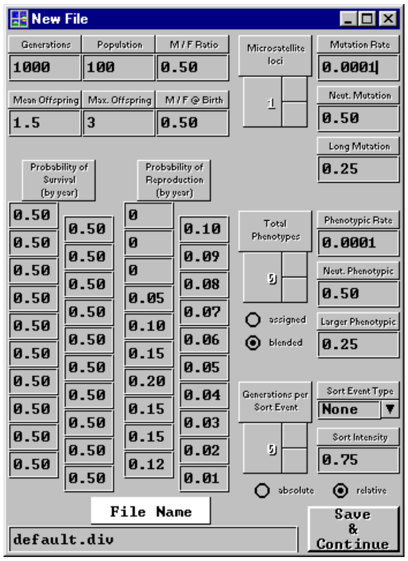
Getting Started

Entering Variables:

Variables can either be entered within a special editing window or loaded in from a text file that follows a specific format. To use the variable editing window in order to enter a new set of variables click on “File” and then on “New Variables” **or** use the shortcut button labeled “New” under the box containing the name of the variable file. (There is no name listed when the program first starts, so only “Variable File:” is visible.)







Generations: 1000
Population: 100
M / F Ratio: 0.50
Mean Offspring: 1.5
Max. Offspring: 3
M / F @ Birth: 0.50
Probability of Survival (by year): 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50, 0.50
Probability of Reproduction (by year): 0, 0, 0, 0.05, 0.10, 0.15, 0.20, 0.15, 0.15, 0.12, 0.10, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01
Microsatellite loci: 1
Mutation Rate: 0.0001
Neut. Mutation: 0.50
Long Mutation: 0.25
Total Phenotypes: 5
Phenotypic Rate: 0.0001
Neut. Phenotypic: 0.50
Larger Phenotypic: 0.25
○ assigned
● blended
Generations per Sort Event: 5
Sort Event Type: None
Sort Intensity: 0.75
○ absolute
● relative
File Name: default.div

Once you are viewing the variable entry and editing window (“New File”) you can enter or change any of the variables listed. The default variable file name is “default.div” and is saved in the same directory or folder as the main Diversion program; however, its location and name can be changed to any valid name or location. If you find yourself in the editing window and wish to leave without changing or saving anything you can click the “X” in the top right corner to close the window.

Variables must be within a specific range of values. Information about what values are acceptable is available by “right-clicking” on the descriptive label above the variable entry box. The box to the right contains a list of limitations for the variables.

Generations	> 1
Population	> 2
M/F Ratio	0<x<1
Mean Offspring	> 0
Max. Offspring	>= Mean
M/F @ Birth	0<x<1
Probability of Survival (by year)	0-1
Probability of Reproduction (by year)	0-1
Microsatellite Loci	1-100
Mutation Rate	0-1
Neut. Mutation	0-1
Long Mutation	0-1
Total Phenotypes	0-100
Phenotypic Rate	0-1
Neut. Phenotypic	0-1
Larger Phenotypic	0-1
“Assigned” or “Blended”	
Generations per Sort Event	0-100
Sort Event Types:	
None	No sort events
Upper	Upper end skew
Lower	Lower end skew
Center	Central values (0) skew
U&L	Upper & lower skewing
U/C/L	Upper, central, & lower skewing
Random	Random elimination
Sort Intensity	0-1
“Absolute” or “Relative”	

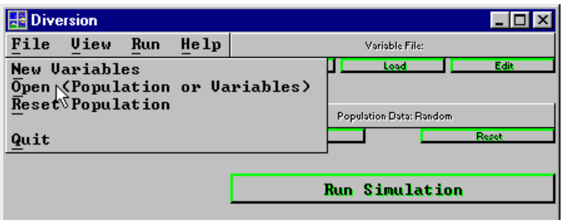
When the “Save & Continue” button is clicked the variables are first checked to be sure that they are all valid (within the limits listed) and then saved as a text file with “.div” as the file extension / type. If there are problems with any entries error messages will appear and the editing window will remain open.

The following is an example of what the variable file looks like and the format that must be used when preparing a text file of variables if the variable editing window is not used:

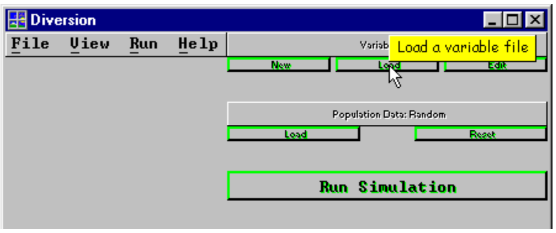
```
.1                     Microsatellite Mutation Rate
 100                   Number of Generations to Process
 1000                  Starting Population Size
 .5                    Male/Female Ratio in Starting Population
 .5                    Proportion of Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Longer Product
 2                     Average Number of Offspring Produced by each Female
 .5                    Male/Female Ratio of Offspring at Birth
 .5                    Probability of Survival from Birth to Year 1
 .8                    Probability of Survival from Year 1 to Year 2
 .9                    Probability of Survival from Year 2 to Year 3
 .95                   Probability of Survival from Year 3 to Year 4
 .95                   Probability of Survival from Year 4 to Year 5
 .95                   Probability of Survival from Year 5 to Year 6
 .9                    Probability of Survival from Year 6 to Year 7
 .9                    Probability of Survival from Year 7 to Year 8
 .8                    Probability of Survival from Year 8 to Year 9
 .7                    Probability of Survival from Year 9 to Year 10
 .6                    Probability of Survival from Year 10 to Year 11
 .5                    Probability of Survival from Year 11 to Year 12
 .5                    Probability of Survival from Year 12 to Year 13
 .5                    Probability of Survival from Year 13 to Year 14
 .5                    Probability of Survival from Year 14 to Year 15
 .5                    Probability of Survival from Year 15 to Year 16
 .5                    Probability of Survival from Year 16 to Year 17
 .5                    Probability of Survival from Year 17 to Year 18
 .5                    Probability of Survival from Year 18 to Year 19
 .5                    Probability of Survival from Year 19 to Year 20
 0                     Probability of Successfully Reproducing at Year 1
 0                     Probability of Successfully Reproducing at Year 2
 0                     Probability of Successfully Reproducing at Year 3
 .3                    Probability of Successfully Reproducing at Year 4
 .4                    Probability of Successfully Reproducing at Year 5
 .6                    Probability of Successfully Reproducing at Year 6
 .6                    Probability of Successfully Reproducing at Year 7
 .6                    Probability of Successfully Reproducing at Year 8
 .6                    Probability of Successfully Reproducing at Year 9
 .6                    Probability of Successfully Reproducing at Year 10
 .5                    Probability of Successfully Reproducing at Year 11
 .5                    Probability of Successfully Reproducing at Year 12
 .4                    Probability of Successfully Reproducing at Year 13
 .4                    Probability of Successfully Reproducing at Year 14
 .3                    Probability of Successfully Reproducing at Year 15
 .3                    Probability of Successfully Reproducing at Year 16
 .2                    Probability of Successfully Reproducing at Year 17
 .2                    Probability of Successfully Reproducing at Year 18
 .1                    Probability of Successfully Reproducing at Year 19
 .1                    Probability of Successfully Reproducing at Year 20
 3                     Number of Microsatellite Loci
 2                     Number of Phenotypes
 0                     Number of Generations to Cycle Through Before Pausing for a Sorting Event
 6                     Maximum Number of Offspring a Female Can Possibly Have
 .1                    Phenotypic Mutation Rate
 .5                    Proportion of Phenotypic Mutations that are Neutral
 .25                   Proportion of Mutations that Produce a Larger Phenotype
assigned               Phenotype Determination Method Used ('assigned' or 'blended')
 .75                   Intensity of Each Sort Event
relative               Method of Calculating Sort Events ('absolute' or 'relative')
C:\WINDOWS\Desktop\Example.div
None                   Type of Sort Event to Simulate ('None', 'Upper', 'Lower', 'Center', 'U&L', 'U/C/L', 'Random'
```

The description of the variable on each line does not need to be included. It is included in the file generated by the program in order to make changes with a text editor (such as Notepad) easier and more accurate. Order of variables listed **must** be as shown and **no** blank or empty lines are allowed at the top, bottom, or anywhere in between.

Loading Variables:



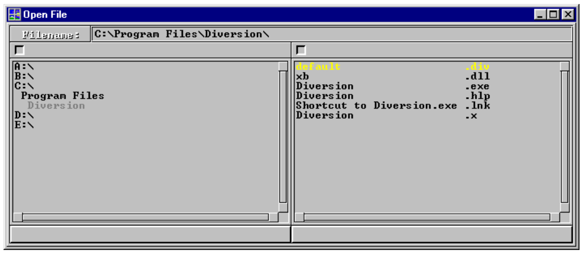
Population Data: Random



Population Data: Random

To load in a set of variables that are already in the accepted format either click on "File" and then "Open (Population or Variables)" **or** use the shortcut button labeled "Load" under the box that contains variable file names.

The window that appears will look something like the window pictured below. If you know the exact location and name of the file you can enter it in the box near the top of the window. Otherwise, you can browse through the directory tree displayed and choose the file by double clicking on it. (Be sure to note that all variable files must end with the file extension type ".div" or an error message will be given.)

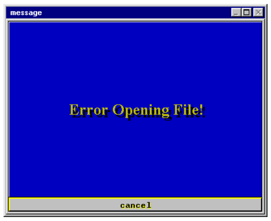


C:\Program Files\Diversion\

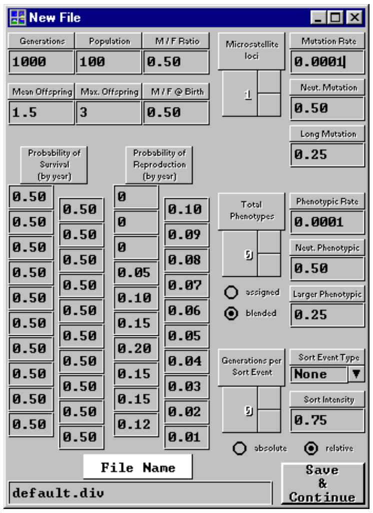
```
A:\
B:\
C:\
 Program Files
  Diversion
D:\
E:\
```

```
default                      .div
xb                           .dll
Diversion                    .exe
Diversion                    .hlp
Shortcut to Diversion.exe    .lnk
Diversion                    .x
```

If there is a problem reading in the file (such as if the file is not found) an error message will be displayed. Right after opening a file all variables are checked for validity (to be sure they are within acceptable ranges). If problems are found an error message will be displayed for each one.



Error Opening File!



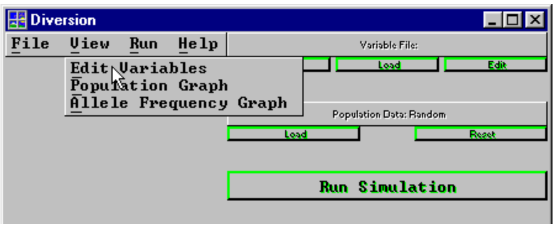
Generations: 1000
Population: 100
M / F Ratio: 0.50
Mean Offspring: 1.5
Max. Offspring: 3
M / F @ Birth: 0.50
Microsatellite loci: 1
Mutation Rate: 0.0001
Neut. Mutation: 0.50
Long Mutation: 0.25

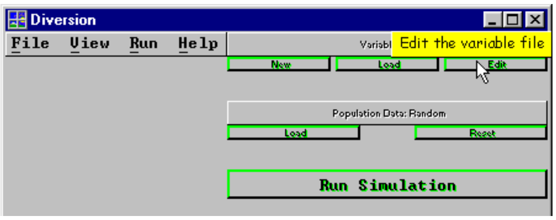
Probability of Survival (by year)		Probability of Reproduction (by year)	
0.50	0.50	0	0.10
0.50	0.50	0	0.09
0.50	0.50	0	0.08
0.50	0.50	0.05	0.07
0.50	0.50	0.10	0.06
0.50	0.50	0.15	0.05
0.50	0.50	0.20	0.04
0.50	0.50	0.15	0.03
0.50	0.50	0.15	0.02
0.50	0.50	0.12	0.01
	0.50		

Total Phenotypes: 5
○ assigned
● blended
Phenotypic Rate: 0.0001
Neut. Phenotypic: 0.50
Larger Phenotypic: 0.25
Generations per Sort Event: 5
Sort Event Type: None
Sort Intensity: 0.75
○ absolute ● relative
File Name: default.div

At the end of variable checking the variable editing window will appear and display the value for each variable read in. Here you can check to be sure everything is as you wish and then click "Save & Continue" to move on. Here you can change the file name for the variables as well, just in case you wish to make some modifications for the current simulation run. If the name of the variable file is not changed the variables displayed will be saved in the same file, replacing anything that the file might have contained originally.

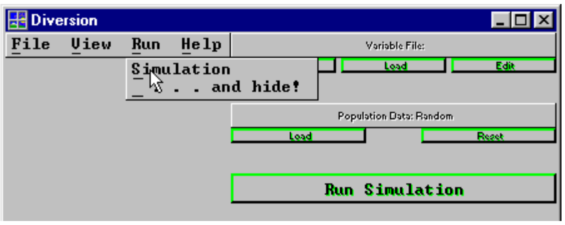
Editing Variables:

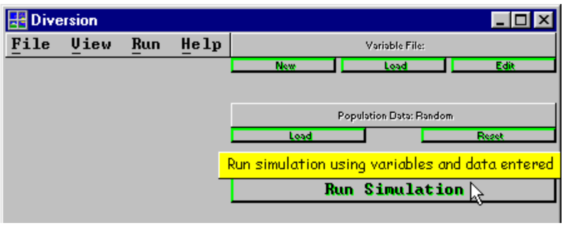




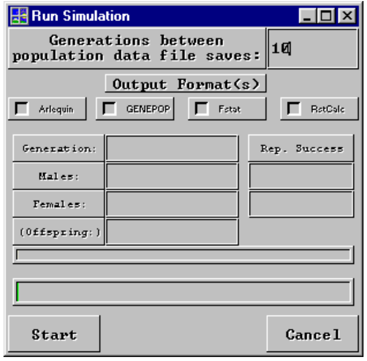
At any time variables can be edited or modified. If no variables have been previously entered or loaded in the default values will appear within the variable editing window. To edit variables you can either click on "View" and then "Edit Variables" **or** use the shortcut button labeled "Edit" under the box that contains variable file names. This is the same window and procedure used to initially enter variables so the same limitations and processes apply. Variables are checked for valid ranges and specific error messages are displayed when problems are identified.

Running the Simulation:





To run the simulation and start calculations either click on "Run" and then "Simulation" **or** use the shortcut button labeled "Run Simulation" in the main program window. When either of these methods is used the first task is to once again check to be sure that all variables are within acceptable ranges. An error message is displayed for each problem found. If any errors are encountered the variable editing window opens, after all errors have been displayed, to allow modification of variables to acceptable values.

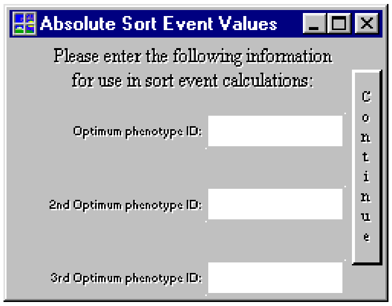


Generations between population data file saves: 10

Once variables are successfully verified the main simulation window appears. Before starting the simulation run you can specify how frequently the population data is to be saved. Population data consists of all the information for each individual within the population listed out in one large file. Two populations (first and last) are always saved. If the value is changed to five (5) then every 5th population is saved into a file. Files are saved into the same directory or folder as the variables file and have the same name with a slight modification. Generation number is added to the end of the variable file name. ".pop" is used as the file extension or type. So the 5th generation resulting from the variables found in “default.div” is labeled “default5.pop” and is saved into the same area as the variable file.

At this time Arlequin’s project format is the only additional output format available from the list of population analysis programs. Putting an “X” in any of the other boxes will not have any effect.

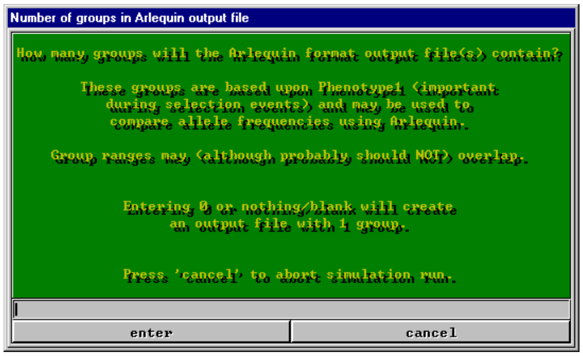
Click “Start” to begin calculations. New windows will appear and a response (or responses) will be required in any active one (with a blue bar across the top). If the sort calculation type is listed as “absolute” then the active window will request phenotype values to use in calculations during sort events. (A selection of “relative” sort events has optimal and most inferior phenotype values calculated automatically based on sort type.) Two to three entries will be required.


Absolute Sort Event Values

Please enter the following information for use in sort event calculations:

Optimum phenotype ID:
2nd Optimum phenotype ID:
3rd Optimum phenotype ID:
Continue

If another output format has been chosen (Arlequin is the only one working at this time) your next entries will be in regard to that format. For the Arlequin output format the first question to be answered concerns how many groups there will be in the Arlequin project file. Arlequin calls these “Samples” and this value is recorded in the project file after the text “NbSamples= ” and followed by the informational comment “#Number of samples in the Project.”



How many groups will the Arlequin format output file(s) contain?

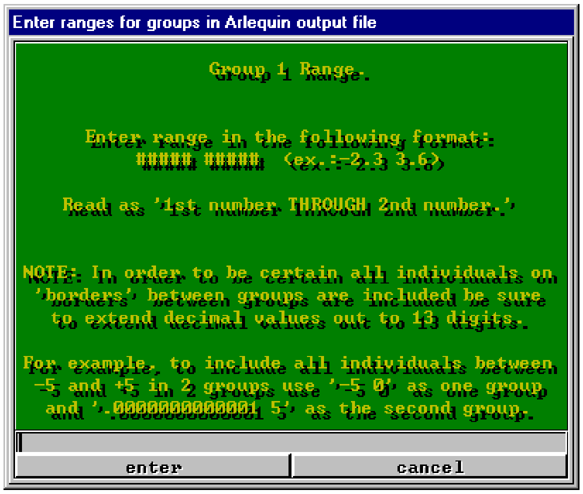
These groups are based upon Phenotype1 (important during selection events) and may be used to compare allele frequencies using Arlequin.

Group ranges may (although probably should NOT) overlap.

Entering 0 or nothing/blank will create an output file with 1 group.

Press 'cancel' to abort simulation run.

After the number of sample groups has been determined you next need to specify what ranges of values, based upon phenotype1, will be included in each “Sample.” The ranges entered can be anything and even overlap (which would probably not be recommended in most cases). The data file produced may be used directly as an Arlequin 2.000 project file. At times you may notice there are fewer “Samples” listed in the project file than you originally specified. Since “Samples” must contain at least one individual, ranges (“Samples”) without individuals are not listed.



Group 1 Range.

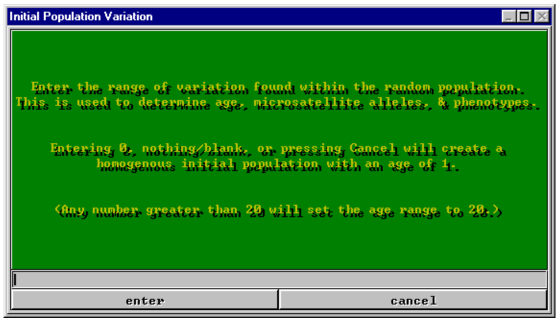
Enter range in the following format:
(ex.: -2.3 3.6)

Read as '1st number THROUGH 2nd number.'

NOTE: In order to be certain all individuals on 'borders' between groups are included be sure to extend decimal values out to 13 digits.

For example, to include all individuals between -5 and +5 in 2 groups use '-5 0' as one group and '.0000000000001 5' as the second group.

NOTE: To ensure that all values between non-overlapping ranges are included you will need to specify out to 13 digits. So if one range is to be from –5 through 0 and another from just above 0 through 5 you would enter "`-5 0`" for the first range and "`.0000000000001 5`" for the second range.



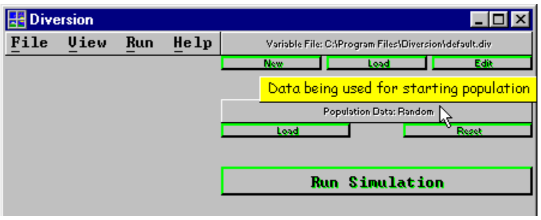
Enter the range of variation found within the random population.
This is used to determine age, microsatellite alleles, & phenotypes.

Entering 0, nothing/blank, or pressing Cancel will create a
homogenous initial population with an age of 1.

(Any number greater than 20 will set the age range to 20.)

If no population has been loaded in a window will appear where you must specify the range of variation present within the initial population. Individuals in the initial population are randomly assigned attributes based on this range. If you enter a 0 or enter nothing and just click on "enter" the entire population will start out with the same microsatellite (and if applicable phenotype) attributes and every individual will have an age of 1. In order to have individuals with ages greater than 20 in the initial population you must manually (with a text editor) create a population file to load in.

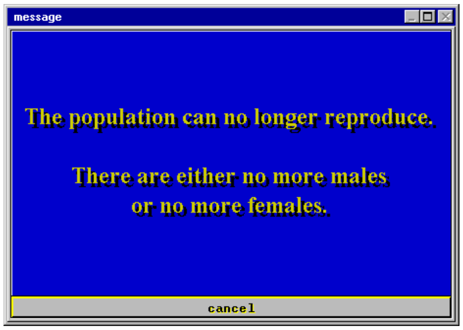
NOTE: This "Initial Population Variation" window will only be displayed if "Population Data:" is set to "Random" (visible in the main program window). If a specific population is loaded in there is no need for the window to be displayed. (Loading a population is discussed below.)



Variable File: C:\Program Files\Diversion\default.div

Data being used for starting population

Population Data: Random

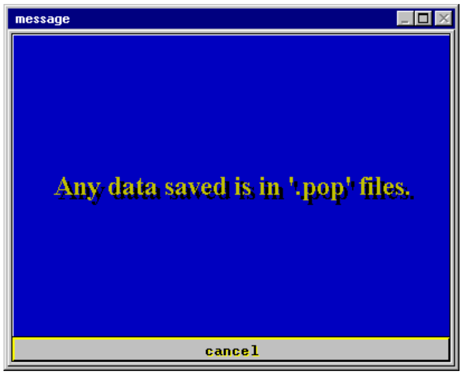


The population can no longer reproduce.

There are either no more males
or no more females.

During the simulation run an error message concerning population size and reproduction may appear. If a population develops that has only males or only females processing stops since further reproduction is impossible.

At the end of processing a message appears stating that population files (ending with “.pop”) have been saved. Any text editor or word processor can be used to view the text files containing population information. Population files are created in a specific format that must be maintained if the files are to be used later to load in initial population information for another simulation run.



Any data saved is in '.pop' files.

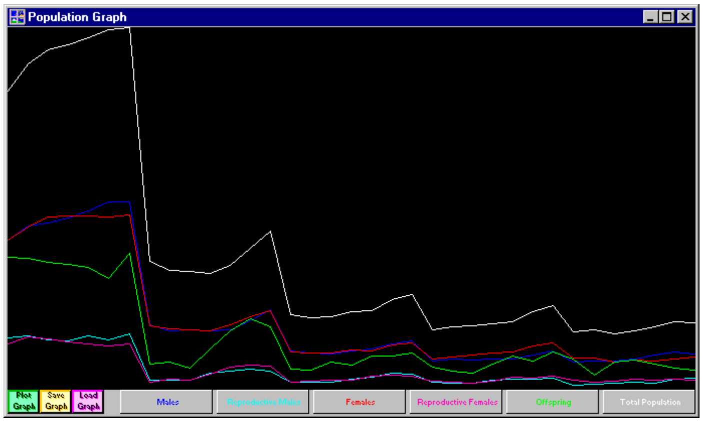
NOTE: When saving a modified file be sure to save it in text (“.txt”) format with “.pop” as the file extension. The file will probably still end in “.txt” (looking something like – MyPopulation.pop.txt) so you will need to change the file name to have .pop as the file type extension by deleting the “.txt” that was added. If a file is opened that ends with “.pop” and then saved again after it has been modified or updated the extension will probably still be “.pop”.

An example of a population file follows:

```
 1                     Total Phenotypes
 1                     Total Microsatellite Loci
 25                    Total Population
Sex   Age             Phenotype1                           MicrosatLocus1
 1      4                     -6                      7                      1
 1      6                     -2                      5                     -5
 1      3                      2                      7                      2
 1      2                     -2                      4                     -2
 1      1                     -4                      4                     -7
 1      2                      5                      5                     -7
 1      1                     -4                      0                     -4
 1      1                      5                      5                      1
 1      2                      7                     -6                     -7
 1      4                      2                     -4                      4
 1      4                     -5                      1                     -4
 1      5                      1                      2                     -1
 2      6                      3                      2                     -1
 2      6                     -5                      0                      3
 2      2                     -6                      6                     -1
 2      2                     -5                      5                      2
 2      6                      1                      2                     -6
 2      6                      0                      4                     -6
 2      7                      2                      2                     -5
 2      6                      0                      0                      4
 2      5                     -5                     -3                      2
 2      7                     -1                      4                      2
 2      2                      7                     -3                     -6
 2      4                     -4                     -7                     -2
 2      2                     -6                     -6                      7
END
```

In the example of population file format above amount of spacing between numbers is not important, but some space is required. Descriptive labels after numbers are not required (phenotypes, microsatellite loci, population size), **but** a line between total population size and the beginning of the list of individuals in the population **is** required. Content of the line is unimportant. The last line of the file must be "END" as indicated. No added spacing between individuals listed is allowed.

Plotting, Saving, & Loading Population Summary Data:



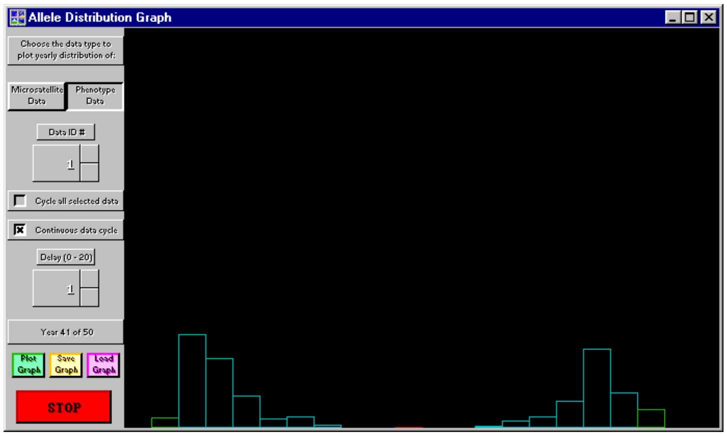
Once a simulation has finished running (or actually even while it is still running) population summary data can be plotted in the “Population Graph” window. The three buttons located at the bottom left corner of the window are used to plot, save, or load previously saved population summary data. A legend indicating what the lines plotted represent fills the remainder of the window’s bottom edge. Allowing the pointer to sit over any legend box for a short time provides a brief description (as is the case for most objects used within the program).

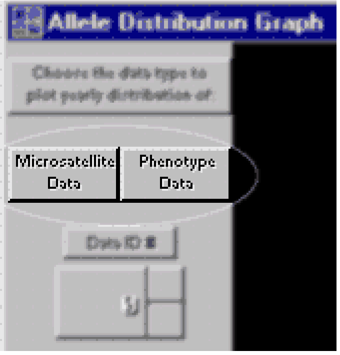
Summary data are saved in text format within a file with the same name as the variable file and a file extension type of “.pps” (indicating **p**o**p**ulation **s**ummary data). The file can be opened and viewed with any text editor or word processor. It is also possible to create population summary data files to load in and have plotted by following the exact same format as is created by the program.

When the “Load Graph” button is clicked the “Open File” window appears. Population summary data has a file extension type of “.pps”. Any other file type used by the program can be loaded as well. There is only one window used to load in data from files. If a population summary data file is not chosen no error message will be displayed. If the file has a valid extension type it will be loaded in (whether it is a variable file, population file, or any other acceptable type). After the data file has been loaded in click the “Plot Graph” button to display the data.

Plotting, Saving, & Loading Allele Distribution Data:

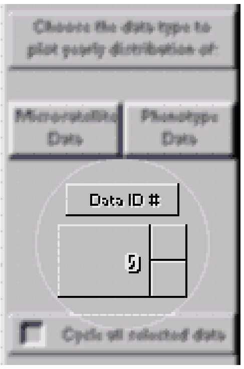
After a simulation run microsatellite allele frequencies and categorized phenotypic attribute distributions can be plotted in the "Allele Distribution Graph" window. Several selections within the window allow the specific data of interest to be displayed.

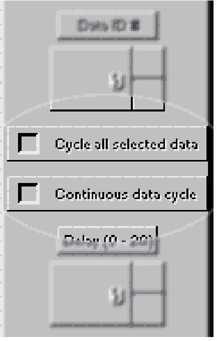



Allele Distribution Graph
Choose the data type to plot yearly distribution of:
Microsatellite Data
Phenotype Data
Data ID #
0

First, the type of data to be plotted must be selected (either microsatellite or phenotype data). A selection is made by clicking on one of the labeled buttons in the top left part of the window. Only one data type can be displayed at a time.

Next, a valid ID # for the data to be displayed has to be chosen. If an invalid ID # is selected an error message is displayed when an attempt to plot the data is made. The box also contains the ID # of the data displayed while it is being displayed (which is important when whole sets of data are being cycled through).

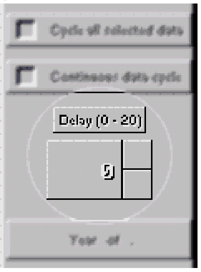

Choose the data type to plot yearly distribution of:
Microsatellite Data
Phenotype Data
Data ID #
0
☐ Cycle all selected data

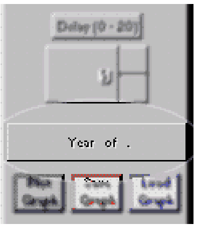


How data are displayed can be chosen now. There are three options. The first option determines how much of the data of the identified type (microsatellite or phenotype) are to be displayed. Default is set to display only the data indicated in the "Data ID #" box. If an "X" is placed in front of "Cycle all selected data" all the data of the type indicated (microsatellite or phenotype) will be cycled through beginning with data ID # 1. If there is an "X" in the box the selection in the "Data ID #" box does not matter. Cycling always begins with ID # 1.

Option number two allows the data displayed to be repeated continuously. If only one set of data is to be displayed that alone is repeated for all generations, however, if all data of the selected type are to be displayed all ID #'s are cycled through for all generations and then cycling begins again with ID # 1. Data cycling continues until the "Stop" button is clicked.

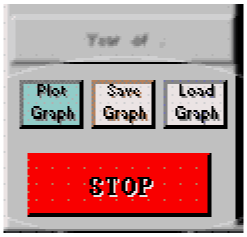
Delay between displays of generational data can also be set. Each number (1-20) represents 1/10 of a second. Selecting 0 will plot data as fast as the computer is capable.





During data plotting the next box in the window displays the current year (or generation) being displayed.

To begin plotting data click the “Plot Graph” button. First a check is made to ensure selections of data to display and how to display it are valid. If problems are detected (or if there are no data available) error messages appear.

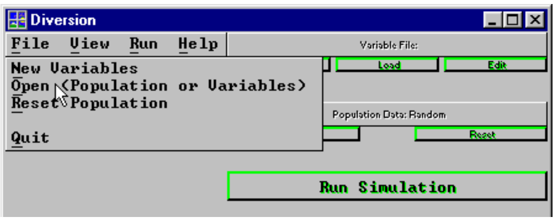


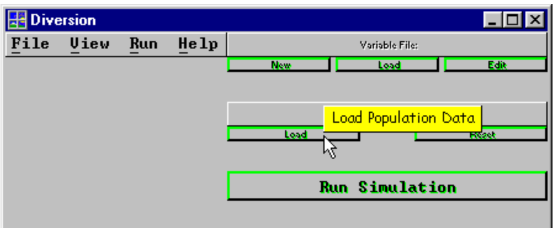
While data are being displayed the process can be terminated by clicking on the “Stop” button. It will always be necessary to use the “Stop” button when data are being cycled continuously.

Data from a completed simulation run can be saved using the “Save Graph” button. A text file containing all necessary data is create with the same name as the variable file, but an extension of “.ppf” (indicating **po**pulation allele **f**requency data) is used. The file can be opened and viewed with any text editor or word processor. It is also possible to create population allele frequency data files to load in and have plotted by following the exact same format as is created by the program.

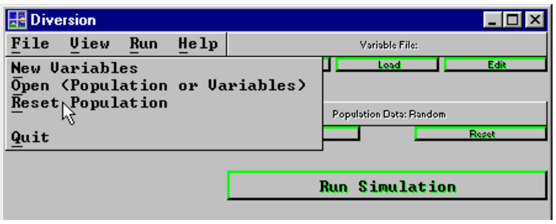
When the “Load Graph” button is clicked the “Open File” window appears. Population allele frequency data has a file extension type of “.ppf”. Any other file type used by the program can be loaded as well. There is only one window used to load in data from files. If a population allele frequency data file is not chosen no error message will be displayed. If the file has a valid extension type it will be loaded in (whether it is a variable file, population file, or any other acceptable type). After the data file has been loaded in set data display values and then click the “Plot Graph” button to display the data.

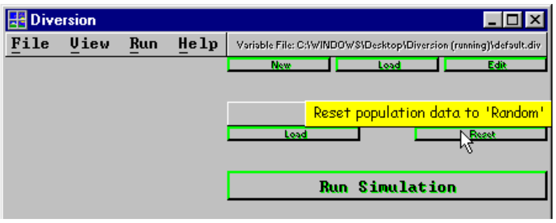
Loading Population File Data:





When a specific starting population is desired an initial population must be loaded in from a file. The format used is the same as the population files (ending with the extension “.pop”) saved during simulation runs. To load a population file either click “File” and then “Open (Population or Variables)” or click on the shortcut button labeled “Load” under the box containing population file descriptive information. All file types are loaded using the same module, so if a “.pop” file is not selected and another valid file type is chosen no error message will be given (as long as the data within the file are OK). If problems are found within a population file error messages are displayed.




Diversion
File
View
Run
Help
Variable File: C:\WINDOWS\Desktop\Diversion (running)\default.div
New
Load
Edit
Reset population data to 'Random'
Load
Reset
Run Simulation

To set the population used for simulation runs back to “Random” either click “File” and then “Reset Population” or use the shortcut by clicking the “Reset” button under the box containing population file descriptive information. Initial population type description will be set to “Random” and variability information will need to be entered right before a simulation starts running.

Details on Specific Calculations and Associated Assumptions

Explanation of Variables Used:

Generations – Total number of generations to cycle through during the simulation run. Valid numbers are integers ranging from 1 through 2147483647.

Population – Size of initial population at generation 1. Valid numbers are integers ranging from 1 through 2147483647, however, 2147483647 individuals is the largest size that any population can reach without creating errors that crash the program. If a population file is loaded this number cannot be changed.

M / F Ratio – Male ratio in the initial population that is randomly set up. Valid values range anywhere between 0 and 1, but cannot be 0 or 1 (since 0 is equivalent to a population containing 0 males and 1 is equivalent to a population with 0 females). If a population file is loaded this number cannot be changed.

Mean Offspring – Mean number of offspring that each reproductively successful female produces. Since this is the mean number of offspring it can be any number (integer or fraction) that is greater than 0. Again, the maximum value allowed is 2147483647, however, practically it is much less. It must always be less than or equal to the Maximum Offspring value.

Max. Offspring – Maximum number of offspring that any single female can produce. This value can range anywhere between 1 and 2147483647, but it must always be greater than or equal to the value entered for Mean Offspring. With extremely large numbers don’t be surprised if there is a quick crash of the program due to an overflow in the population counter (which itself can only go up to 2147483647).

M / F @ Birth – Male ratio in offspring calculations. This value is used to randomly determine whether offspring will be male or female. Valid entries range between 0 and 1, but cannot be 0 or 1 (since 0 is equivalent to all offspring being females and 1 is equivalent to all offspring being males).

Probability of Survival (by year) – Probability that an individual of the given age will survive until the next year (generation). Acceptable values range from 0 (no possibility of survival) through 1 (100% survival). For all ages greater than 20 the value for age 20 is used.

Probability of Reproduction (by year) – Probability that an individual of the given age will be reproductively successful in the current year (generation). Acceptable values range from 0 (no possibility of reproductive success) through 1 (100% reproductive success). For all ages greater than 20 the value for age 20 is used.

Microsatellite Loci – Total number of microsatellite loci to keep track of. This value may be incremented from 1 up through 100, but must always be at least 1.

> **Mutation Rate** – Rate that mutations occur in microsatellite alleles. Acceptable values range from 0 (no mutations ever occur) through 1 (every allele experiences mutation in each generation and is passed on to offspring).
>
> **Neut. Mutation** – Rate at which neutral mutations occur in microsatellite alleles. Acceptable values range from 0 (no neutral mutations ever occur) through 1 (every allelic mutation is neutral). A neutral mutation doesn't change microsatellite allele value (which is equivalent to fragment size on a gel). The sum of neutral mutations and long mutations must be less than or equal to 1. Rate of shortening mutations is what remains after subtracting neutral and long mutation rates from 1.
>
> **Long Mutation** – Rate at which mutations extending the length of a microsatellite allele occur. Acceptable values range from 0 (no lengthening mutations ever occur) through 1 (every allelic mutation extends the length of the allele). The sum of neutral mutations and long mutations must be less than or equal to 1. Rate of shortening mutations is what remains after subtracting neutral and long mutation rates from 1.

Total Phenotypes – Total number of phenotypes to keep track of. This value may be incremented from 0 up through 100.

Phenotypic Rate – Rate that mutations occur in phenotypes. Acceptable values range from 0 (no mutations ever occur) through 1 (every phenotype experiences mutation in each generation and is passed on to offspring).

Neut. Phenotypic – Rate at which neutral mutations occur in phenotypes. Acceptable values range from 0 (no neutral mutations ever occur) through 1 (every phenotypic mutation is neutral). A neutral mutation doesn't change phenotype value. The sum of neutral mutations and larger mutations must be less than or equal to 1. Rate of smaller mutations is what remains after subtracting neutral and larger mutation rates from 1.

Larger Phenotypic – Rate at which mutations producing larger phenotypes occur. Acceptable values range from 0 (no enlarging mutations ever occur) through 1 (every phenotypic mutation enlarges the phenotype). The sum of neutral mutations and larger mutations must be less than or equal to 1. Rate of smaller mutations is what remains after subtracting neutral and larger mutation rates from 1.

Offspring phenotype determination method – Select how offspring phenotypes are calculated or assigned.

- **assigned** – One parental phenotype is randomly selected and assigned to an individual offspring. Each offspring receives a different randomly chosen parental phenotype.
- **blended** – Offspring phenotype is calculated as the mean of parental phenotypes. For example, parents with phenotypes 4 and 6 produce all offspring with phenotype 5.

Generations per Sort Event – Number of generations between sort events. When sort events are to occur this value must range between 1 and 100.

Sort Event Type – Selection of what type of periodic sort events are to occur.

- **None** – No sort events are ever calculated.
- **Upper** – Sort events skew phenotype1 selection toward larger sizes.
- **Lower** – Sort events skew phenotype1 selection toward smaller sizes.
- **Center** – Sort events skew phenotype1 selection toward central (median) sizes.
- **U & L** – Sort events skew phenotype1 selection toward the opposite extremes (larger and smaller).
- **U/C/L** – Sort events skew phenotype1 selection toward larger, central, and smaller sizes.

Random – Sort events eliminate individuals randomly, so phenotype1 has no influence on survival.

Sort Intensity – Strength of selection events. Acceptable values range from 0 (no selection) through 1 (most intense selection).

Sort event calculation method – select how sort events are calculated.

absolute – specific values are set as optimum and most inferior phenotypes for phenotype1.

relative – optimum and most inferior phenotypes for phenotype1 are calculated for each generation based upon the sort event type chosen.

Calculations Made and Associated Assumptions:

Initial Population Setup – Initial random population setup is performed if no specific starting population has been loaded in. Range of variation is based on the number entered in the initial population variation window. The absolute value of the entered number is determined and all microsatellite and, if applicable, phenotype values for each individual in the population are chosen from within that range on either side of and including zero. Age is selected randomly from a range of 1 through the absolute value of the number entered. If the absolute value entered is greater than 20 age is set to randomly be chosen from between 1 and 20.

Male / Female Sort – This subroutine sorts males and females within the population into separate groups. During the process information on allele frequencies and ranges for each microsatellite and, if applicable, phenotype is kept track of and stored in arrays for later plotting or storage as a text file.

Male / Female Reproductive Success – This subroutine goes through each individual within the population and based upon the age related reproductive success variables randomly determines whether individuals are reproductively successful in the current year / generation. Reproductively successful males are kept track of in a secondary array listing all those that are reproductively successful. This secondary array is used next when choosing what male to match up with a particular female.

Reproduction – Here is where offspring for reproductively successful individuals within the population are calculated. The process goes through all females and those that have been identified as reproductively successful in the current year are used to calculate offspring.

Offspring Calculations – First total number of offspring for a reproductively successful female is calculated. If that number is at least 1 then a reproductively successful male is chosen from the pool. After parents have been chosen and number of offspring is known then alleles are assigned and sex determined for each offspring produced.

Total Offspring – Total offspring that a female will have is determined randomly based upon the variables given for the mean number of offspring and maximum number of offspring. Range for random values is from 0 through the maximum. First a random number is chosen to determine whether the number of offspring will be above or below the mean. If the random number is below or equal to *Percentage@OrBelowMean* in the following equation then number of offspring is randomly chosen from within the range 0 through *MeanOffspringPerFemale*, otherwise, number of offspring is randomly chosen from within the range *MeanOffspringPerFemale* through *MaximumOffspringPerFemale*.

$$Percentage@OrBelowMean = 1 - \left(\frac{MeanOffspringPerFemale}{MaximumOffspringPerFemale} \right)$$

Selection of Male – A male is randomly selected from the array of reproductively successful to father all of a female’s offspring. Because a male is randomly selected for each female processed individual males identified as reproductively successful may father multiple times or not at all.

Assignment of Alleles – All microsatellite alleles are assigned in the same way, however phenotypes can be assigned in two possible ways.

Microsatellite Alleles – One allele is randomly selected from the female and one from the male for each microsatellite locus for each offspring.

Phenotypes – If phenotype determination is set to “assigned” each offspring phenotype is chosen by randomly assigning either the male or female phenotype of the same ID#. If phenotype determination is set to “blended” each offspring phenotype is assigned the mean of the two parental phenotypes. Using “blended” phenotype assignment results in all offspring having the same phenotype.

Sex Assignment – Based on the “M/F @ Birth” variable sex is randomly assigned to each offspring.

Survival – At the end of each generation survival to the next generation is determined. Standard survival calculations based on the yearly survival variables are made. If a periodic sort event is to occur those calculations are made for each individual as well. Attribute information for each surviving individual in the population is saved in a pooled population array.

Yearly Survival – If a randomly chosen number between 0 and 1 is below the variable value for a given age an individual survives and attribute information (microsatellite and phenotype allele values, sex, and age) is copied into the total population array. The total population array contains all individuals that will be present in the next generation.

Sort Event Calculations – During generations with sort events each individual that survives initial yearly survival calculations is further evaluated based on phenotype1 attribute value. Based upon the type of sort event phenotype1 values leading to highest and lowest survival are determined. A relative quality value (from 0 to 1) is assigned to each individual based on phenotype1 value. To determine whether an individual will survive the sort event a random number between 0 and 1 is selected and multiplied by the sort event intensity variable (that also ranges from 0 to 1). If this value is less than or equal to the relative quality previously assigned the individual survives.

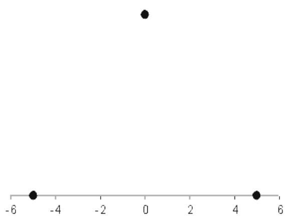
Relative Value Calculation – The following formula is used to assign relative survival value to each individual based upon phenotype1 value.

$$IndividualSurvivalValue = \frac{Worst - \left|Best - Phenotype1\right|}{Worst}$$

For assignment of **Worst** and **Best** values see the following descriptions of sort events.

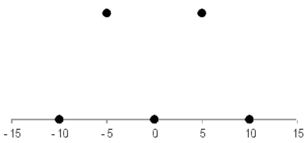
Sort Event Types – Determination of what values for phenotype1 are considered most adaptive (so the individual is most likely to live) and least adaptive (making the individual most likely to die) are made based upon variables provided. Two methods are possible. If absolute/constant values are to be used those values are entered when prompted, just before the simulation begins to run. If relative values are used then for each generation the numbers are recomputed based on the range of phenotype1 values found within the population.

Absolute – If absolute unchanging optimum (and, if applicable, most inferior) values are entered prior to a simulation run the following methods are used to calculate *Best* and *Worst* values for specific sort event types.



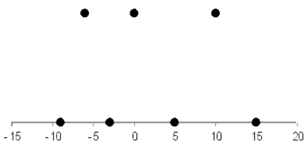
Upper – Best = optimum entered prior to simulation run. Worst (2 values) = Best ± absolute value of the distance between optimum and most inferior entered prior to simulation run. (See figure to the left.)

Lower – Best = optimum entered prior to simulation run. Worst (2 values) = Best ± absolute value of the distance between optimum and most inferior entered prior to simulation run. (See figure to the left.)



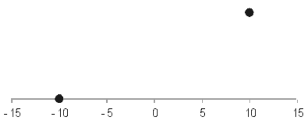
Center – Best = optimum entered prior to simulation run. Worst (2 values) = Best ± absolute value of the distance between optimum and most inferior entered prior to simulation run. (See figure to the left.)

U&L (Upper & Lower) – Best = 2 optimums entered prior to simulation run. Worst (3 values) = 2 optimums ± mean of the 2 optimums. (See figure to the left.)

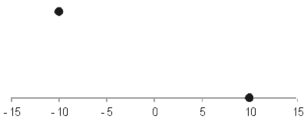


U/C/L (Upper/Center/Lower) – Best = 3 optimums entered prior to simulation run. Worst (4 values) = optimum ± mean distance to next optimum. Halfway between optimums is worst. (See figure to the left.)

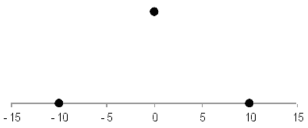
Relative – If relative optimum and most inferior values are used then *Best* and *Worst* values are calculated according to the following methods, based on the sort type.



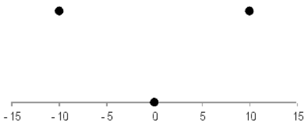
Upper – Best = most positive phenotype1 value found within the current population. Worst = most negative phenotype1 value found within the current population. (See figure to the left.)



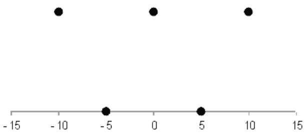
Lower – Best = most negative phenotype1 value found within the current population. Worst = most positive phenotype1 value found within the current population. (See figure to the left.)



Center – Best = mean of the most positive and most negative phenotype1 values found within the current population. Worst (2 values) = most positive and most negative phenotype1 values found within the population. (See figure to the left.)



U&L (Upper & Lower) – Best (2 values) = most positive and most negative phenotype1 values found within the population. Worst = mean of the most positive and most negative phenotype1 values found within the current population. (See figure to the left).



U/C/L (Upper/Center/Lower) – Best (3 values) = most positive, most negative, and mean of the most positive and most negative phenotype1 values found within the population. Worst (2 values) = halfway between optimums. (See figure to the left.)

Random – Random sort events eliminate individuals from the population based only upon selection strength and a random number generated for each individual.

Programming Language and Source

Diversion was programmed using XBasic 6.2.2 (originally created by Max Reason), a freely available open source programming language. Full source code of Diversion (Diversion.x) is included in the compressed distribution. Any changes desired may be made and the program can then be recompiled into a working executable for both Windows and Linux. If changes are incorporated and further distribution is made all changes should be clearly identified and stated.

Information on and access to the XBasic programming language is available at the following web sites:

General information and access to files - **http://www.xbasic.org/**
Official host of XBasic open source project - **http://xbasic.sourceforge.net/**
Good assistance in programming - **http://groups.yahoo.com/group/xbasic**
Online tutorial - **http://gnetools.sourceforge.net/xbsupport/index.html**
Original author’s site - **http://www.maxreason.com/software/xbasic/xbasic.html**
A few XBasic resources - **http://www.xbasic.net/**